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THE UNIVERSITY OF ALBERTA

CALCIUM UPTAKE AND ENZYMATIC ACTIVITIES OF SUBCELLULAR FRACTIONS FROM CARDIOVASCULAR SYSTEM OF NORMOTENSIVE AND HYPERTENSIVE RATS

(CALCIUM REGULATION AND HYPERTENSION)

BY

JIANN-WU WEI ©

A THESIS

SUBMITTED TO THE FACULTY OF GRADUATE STUDIES AND RESEARCH
IN PARTIAL FULFILLMENT OF THE REQUIREMENTS FOR THE DEGREE
OF DOCTOR OF PHILOSOPHY

DEPARTMENT OF PHARMACOLOGY

EDMONTON, ALBERTA

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THE UNIVERSITY OF ALBERTA

FACULTY OF GRADUATE STUDIES AND RESEARCH

The undersigned certify that they have read, and recommend to the Faculty of Graduate Studies and Research, for acceptance, a thesis entitled "Calcium Uptake and Enzymatic Activities of Subcellular Fractions from Cardiovascular System of Normotensive and Hypertensive Rats" submitted by Jiann-Wu Wei in partial fulfillment of the requirements for the degree of Doctor of Philosophy.

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ABSTRACT

1. The altered contractile function of small blood vessels in the hypertensive state is not understood. Genetically determined spontaneous hypertension in rats is a well-studied model of human hypertension. Alterations in the control of intracellular calcium ion may account for altered contractile functions in arteries from these rats. The techniques developed to study control of intracellular calcium by isolated cell organelles along with other biochemical changes in organelle functions are discussed.
2. Fractions enriched in plasma membrane (PM), endoplasmic reticulum (ER),or mitochondria (MIT) were isolated from mesenteric arteries, aortae and myocardium of spontaneously hypertensive rats (SHR) and from two strains of normotensive Wistar controls (NWR). Differential centrifugation and discontinuous sucrose gradients were used.
3. In all tissues, PM fractions had higher activities than did other fractions of enzymes (5′-nucleotidase, Mg^{++}-ATPase, Ouabain-sensitive K^{+}-phosphatase, alkaline phosphatase, phosphodiestrase I, etc.) believed to be markers for sarcolemma; however the extent of enrichment in markers varied and was least in aortae.
4. Calcium uptake and enzymatic activities of PM, SR and MIT fractions from ventricles of SHR and NWR rats were similar. Ouabain-sensitive K^{+}-phosphatase activity was greater in the PM fraction from SHR animals.
5. Calcium uptake in the presence of ATP by the PM fraction from SHR aortae was significantly lower than that from NWR, while the specific activities of 5′-nucleotidase and phosphodiesterase I were greater in the hypertensive than in normotensive fractions.

6. The specific activities of PM marker enzymes were significantly greater in the PM fraction from mesenteric arteries of SHR than in those from NWR. Activities of alkaline phosphatase and Mg^{++}-ATPase were increased in all fractions of arteries from SHR as compared to those from NWR. The maximum calcium uptake in the presence of 5 mM ATP and 17 μM free calcium by the PM fraction from SHR arteries was significantly greater than that from NWR. In contrast, the maximum ATP-independent calcium uptake in the presence of 1 mM free calcium by the PM fraction from SHR arteries was significantly lower than that from NWR. Calcium uptake by membranes from NWR and SHR arteries also differed in their sensitivities to ionophores and multivalent cations (Sr^{++}, Ba^{++}, Mn^{++}).

7. In PM and ER fractions from mesenteric arteries of young (33 days), pre-hypertensive SHR or of early hypertensive (3-4 months) rats, enzyme activities were generally similar to those of similar fractions of NWR. When hypertension was fully developed (5-6 months), most of these values had declined in NWR but not in SHR. Alkaline phosphatase activities of PM fractions from arteries of SHR were elevated at 33 days compared to values from age-matched NWR and the differential increased with age and increasing hypertension. Calcium uptake by fractions from these arteries did not change with age in NWR and was higher in SHR only at 5-6 months.

8. The following conclusions were drawn:

   a) Biochemical changes in arteries are associated with hypertension.

Only increased alkaline phosphatase activity had a pattern of change which might be related to the pathological process that initiated hypertension. Other changes may have been secondary to hypertension or related to change in pathology which occurs with persisting hypertension.

b) The total pattern of change is not explicable as a result of changes resulting from increased wall thickness of arteries.

c) The differences between aortae and mesenteric arteries in the changes associated with hypertension show that the aorta may not be an appropriate model for small arteries.

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CHAPTER I

GENERAL INTRODUCTION

CHAPTER I: GENERAL INTRODUCTION

A) Animal Model Chosen in this Study

Essential hypertension is the commonest and most important entity of the hypertensive diseases, it comprises roughly 90% of hypertension in human beings. Estimates made by the National Health Survey in 1962 indicate that about 26 million persons in the United States have hypertension or hypertensive heart disease. When hypertension is defined in terms of blood pressure readings of 150/100 mm Hg or greater, 20 percent of men and women aged 45 to 54 years are found to be afflicted (Gordon and Devine, 1966) Various kinds of animal models have been used to study the pathogenesis of essential hypertension and to establish measures for its prevention.

Okamoto and Aoki (1963) developed a colony of rats that exhibit hypertension spontaneously without exception. These animals were called Spontaneously Hypertensive Rats (hereafter abbreviated as SHR). The hypertension exhibited by SHR appears to be analogous to essential hypertension in humans. This strain of rats is considered to be the most appropriate animal model available for essential hypertension in man. The aim of the present investigation was to determine whether or not any of the biochemical alterations are present in the cardiovascular system of SHR. Interests were focused on calcium uptake and certain enzymatic activities in vascular smooth muscle at the subcellular level. Altered regulation of calcium metabolism is a plausible cause of vascular smooth muscle supersensitivity while alterations in certain enzymes may reflect cellular changes associated with the initiation of, or the responses to, hypertension.

B) Etiology of Human Essential Hypertension and Comparison to Hypertension in SHR

Mean arterial pressure is determined by cardiac output and peripheral resistance. An increased pressure may be induced by increases of one, or both, of these factors. These possibilities, as they relate to arterial pressure changes in essential hypertension, will be briefly outlines: √

(i) Cardiac output

It has been suggested that an increased cardiac output might play an important part in the initiation of essential hypertension. Thus, Wezler and Böger (1939), and Varnauskas (1955) observed an increased cardiac output in combination with a largely normal peripheral resistance in hypertensive subjects. These findings have been further confirmed and suggested to be present in the early phase of the disease (e.g. Lund-Johansen, 1967; Frohlich et al., 1970, Julius and Schork, 1971). On the other hand, there is no evidence of an increased cardiac output in the established phase of hypertension (Freis, 1960; Pickering, 1968). Similar conclusion of SHR has been reported (Pfeffer and Frohlich, 1973). Furthermore, patients with increased cardiac output caused by a number of disorders do not develop hypertension (Page, 1974). Most subjects in the established phase of hypertension have an increased peripheral resistance and normal or decreased cardiac output.

(ii) Peripheral resistance

According to Poiseulle's law the ratio of pressure drop to flow is a function of all the forces that retard blood flow - the viscosity of blood and the length and radius of the vessels. This ratio has been adopted as an expression of vascular resistance, by analogy with Ohm's law for electrical circuits. The resistance is proportional to the length of the resistance vessels and to blood viscosity, and inversely proportional to the fourth power of the internal radius of the vessel. Therefore, changes in the internal radius have a far greater influence on resistance to flow than changes in either viscosity or vascular length. No studies have suggested that an increase in length of the vessel affecting systemic resistance or a raised blood viscosity play an important role for the increase in resistance in essential hypertension (Pickering, 1968). Therefore, most investigators have been concerned with decreases of the internal radius of resistance vessels. Several possible mechanisms that may enhance vascular constriction and lead to decreased radius of blood vessels have been proposed.

(a) Enhanced neural activity

There is normally a tonic discharge of the sympathetic nerves to the small vessels maintaining them in a state of partial constirction. The activity of these vasoconstrictor nerves is co-ordinated mainly in the vasomotor center (V.M.C.). Various regions of this V.M.C. affect the vasoconstrictor tone and hence the arterial pressure change in the activity of vasoconstrictor nerves. It is thought that the sympathetic vasodilator nerves play only a minor role in the regulation of arterial pressure, except during exercise.

Studies on essential hypertension with human and animal models have led to the view that the sympathetic nervous system plays an important role in the maintenance of a certain level of blood pressure in hypertensive subjects as well as in normotensive individuals (Pickering, 1968). This has been supported by the results of immunological sympathectomy (Dorr and Brody, 1966; Smirk, 1970), administration of sympatholytic drugs (Okamoto et al., 1966) or ganglion blocking agents (Okamoto et al., 1966; Okamoto, 1969; Smirk, 1970). These and related findings have led to suggestions of enhanced sympathetic activity in hypertension. However, some discrepancies with this hypothesis were also noted. For example, a decrease in catecholamine turnover in peripheral tissue was found (Louis et al., 1970; Nakamura et al., 1971; Yamori et al., 1972; Sjoerdsma, 1972); as was a decreased tyrosine hydroxylase activity in the vascular system of hypertensive subjects as compared to that of normotensive ones (Traver et al., 1971). No unequivocal agreement has been reached as to whether sympathetic nervous activity is increased in hypertension.

On the other hand, an increased threshold level of blood pressure for baro-receptor nerve activity to decrease the sympathetic activity in hypertensive animals was noted as compared to that of normotensive ones (Kezdi and Wennemark, 1958; Nosaka and Wang, 1972). Since the cardiac output is normal in hypertension, the resetting of baro-receptors is considered the secondary effect of hypertension (Nosaka and Wang, 1972). An explanation for such a resetting may be that the baro-receptor nerve endings of SHR become protected against excessive stimulation during hypertension by structural changes in arteries where these receptors are located. Since the intimal and medial layers of carotid arteries and the aortic arch of SHR are hypertrophied, the transmission of the pressure signal through the medial smooth muscle is dampened to a larger extent in SHR (Wellens et al., 1975).

In summary, the sympathetic nervous system plays a role in the maintenance of a certain level of blood pressure, both in normotensive and hypertensive animals, but there is little evidence that a functional change in this system is the cause of sustained essential hypertension.

(b) Increased humoral vasoconstrictor or decreased vasodilatory agents

Increased amounts of circulating substances which may directly or indirectly cause hypertension have been found in some forms of hypertensive disease, e.g. noradrenaline in phaeochromocytoma, adrenal corticoids in Cushing's syndrome and Conn's disease, and renin-angiotension in renal and malignant hypertension. On patients with uncomplicated essential (primary) hypertension, however, increased amount of circulating pressor substances have not been convincingly demonstrated. Currently, no convincing evidence indicates that humoral agents such as angiotensin, bradykinin, prostaglandins or unknown substances from kidney or other organs are involved in the mechanisms of essential hypertension. The role of adrenal medulla in the mechanisms of hypertension also has been evaluated. Both increased (Ozaki et al., 1972; Nagatsu et al., 1972) and decreased (Slotkin and Green, 1975) levels of synthesis of catecholamine from SHR as compared to that of normotensive rats have been reported.

In summary, there is no convincing evidence for an increased vasoconstrictor substance in either human essential hypertension or in genetic hypertension in rats.

(c) Increased vascular reactivity

The following terms are used differently by various authors and in order to avoid confusion, the most accepted definitions are stated below: It is defined that the terms "supersensitive" and sometimes "hypersensitive" are used to described a tissue (e.g. smooth muscle) in which the amount of a substance required to produce a very small or minimal biological response is less than that of a normal or "control" tissue. The portion of the dose-response curve near threshold doses of a supersensitive tissue lies to the left of that for the "control". The terms "hyperreactivity", "hyperresponse" and "increased reactivity" refer to a greater response to a given dose by some tissue relative to a control. The response may be any parameter measured. These terms include supersensitive tissues but also describe tissues with increased maximal contractile response or pressure or blood flow response. These terms also describe the case of an increased steepness of the dose response curve without a change in threshold dose.

(i) In Vivo

A variety of experimental techniques have been used to demonstrate an increased reactivity of blood vessels to contractile stimulants of patients with essential hypertension. Kaplan and Silah (1964) found that patients with essential hypertension required half as much angiotensin to produce a given rise in blood pressure as did normotensive individuals. Doyle et al. (1959) studied the response of the forearm circulation to norepinephrine, angiotensin and 5-hydroxytryptamine infused into the brachial artery. They found that at the same doses hypertensive patients responded to norepinephrine and 5-hydroxytryptamine with a greater vasoconstriction than did normotensive patients. An in vivo study by Okamoto et al. (1966) reported a greater vascular responsiveness to norepinephrine in SHR.

(ii) Perfusion study

Haeusler and Haefely (1970) found an increased responsiveness (both threshold and maximal responses) of SHR perfused mesenteric arteries to agonists (noradrenaline and KCl). However, the increased vascular reactivity of hypertension by the techniques of in vivo or in vitro perfused blood vessels may result from two possible mechanisms: (a) It may be due to some structural alteration of blood vessels resulting in a vascular reactivity change; (b) It may be due to changes in sensitivity of the vascular smooth muscle cells.

From perfusion studies with several vascular beds, Folkow (1971) and Folkow et al. (1970) have concluded that there is no change in sensitivity of vascular smooth muscle in SHR and that a structural change (increased wall thickness to lumen size ratio) is responsible for the increase in total peripheral resistance. The basis of their argument is that concentration-response curves of vascular resistance to norepinephrine for hypertensive animals differed from those for normotensive animals in the same way as did the calculated concentration-response curves of a mathematical model in which it was assumed that medial thickness had increased by 30% and the increase in wall thickness had encroached on the lumen when the smooth muscle was completely relaxed. If so, responses to all constrictor agents should be equally enhanced in hypertensive blood vessels. In contrast, Lais and Brody (1975) found that the threshold dose for norepinephrine was lower in perfused hindquarters of SHR. The failure of Folkow to obtain an increased sensitivity to norepinephrine may be due to his use of an artificial medium for perfusion or to his use of papaverine treatment. Other investigators (Haeusler and Finch, 1972; Hinke, 1966; McGregor and Smirk, 1970; McQueen, 1956), using perfused preparations from SHR and deoxycorticosterone acetate (DOCA) hypertensive rats, have found increased vascular reactivity to various stimulating agents. McGregor and Smirk (1970) noted that the vasoconstrictor responses to norepinephrine, angiotensin, and 5-hydroxytryptamine in arteries from genetic hypertensive rats exceeded those observed in arteries from controls. One of the techniques they used to obtain their results was the measurement of perfusion pressures in isolated perfused mesenteric arteries. However, they found a greater increase in sensitivity to 5-hydroxytryptamine than to norepinephrine in this isolated preparation from normal and hypertensive rats. McGregor and Smirk concluded that the enhanced response of the diseased arteries was due to an increased sensitivity of the smooth muscle cells rather than to a structural difference between the normal and hypertensive arteries. Similar results were reported by Haeusler and Finch (1972).

It is important to emphasize that an increase in vascular reactivity indicated by a greater pressor response of a perfused vascular bed does not, in itself, permit a differentiation between increased wall thickness and increased sensitivity of vascular smooth muscle. However, the contractile responses of helical strips of the blood vessel wall can be interpreted more directly in terms of sensitivity of vascular smooth muscle.

(iii) Vascular Strips

In many studies, the maximal contractile responses of strips of aortic or femoral arteries from hypertensive animals have been found unchanged or decreased in response to vasostimuli (Spector et al., 1969; Hallback et al., 1971; Massingham and Shevde, 1971; Shibata et al., 1973; Field et al., 1972), but a lower threshold to norepinephrine and potassium chloride (Holloway and Bohr, 1973; Field et al., 1972) has also been found in some but not all studies. Differences can be attributed to a number of factors: type and duration of hypertension, species, age, tissue studied, and differences in experimental technique. A few points should be noted: (1) The studies of aortic and femoral strips do not necessarily reflect the functional characteristics of smooth muscle of the resistance vessels which is the vascular section of interest. (2) It is not known whether the threshold response, the slope of the dose-effect curve, or the maximal response to stimuli is closely related to the determinant of hemodynamic response. (3) However, it is clear that reactivity of both large and small vessels is changed and that supersensitivity to at least some agents is seen in both.

According to the analysis of Johansson (1974) on the possible mechanisms involved in an altered reactivity in hypertension, the mobility of calcium and the delivery of ATP to the contractile machinery are the most susceptible steps. An attractive question that is worthy of testing is: are these steps altered in hypertensive disease?

C) Changes in Cellular or Subcellular Metabolism in Vascular Smooth Muscle of Hypertensive Animals

Changes of certain parameters in cellular or subcellular levels have been found in hypertensive animals which may be directly or indirectly involved in an increase of vascular reactivity. Below, some important parameters are outlined.

(i) Changes in vascular electrolyte content

A marked increase in Na^+ , K^+, and water content in arteries of various types of experimental hypertensive animals (Tobian, 1956; Daniel and Dawkins, 1957; Willard, 1968; Koletsky et al., 1959) and the late phase of SHR (Nagaoka, 1970) have been reported. The increased cation content is believed to be due to binding to extracellular membrane-mucopolysaccharides, which are altered at the hypertensive stage (Headings et al., 1960). However, there has been considerable difficulty in attributing altered electrolyte content to the extracellular fluid, the extracellular matrix or to intracellular loci. Jones (1973, 1974) reported that an increase in membrane permeability to Na^+, K^+ and Cl^-, based on a study of their turn-over rate, was present in SHR large and small arteries; but he found no changes in the total amount of these ions present. Friedman (1974) has found that vascular smooth muscle cell membrane in rats with DOCA-induced hypertension shows a significant increase in "leakiness" as indicated by a fivefold increase in the transmembrane passage of Li^+ and Na^+ at 2°C. Therefore, the increase of these cations in hypertensive arteries may be due to a partially intracellular binding. In the early stage of a study of hypertension, Friedman et al. (1959) have proposed that the sodium concentration gradient between the outside (Na_o^+) and inside (Na_i^+) of the smooth muscle cell is a basic determinant of tone, an increase in gradient (Na_o^+/Na_i^+) leading to a decrease in tone, a decrease in gradient to an increase tone. The blood pressure setting from low to high values is viewed as depending on graded changes in sodium transfer equilibria.

The concentration of the ion more intimately involved with regulation of contraction, calcium, is reported to be unchanged (Beilin et al., 1969; Douglas et al., 1967) or slightly increased (Tobian and Chesley, 1966; Massingham and Shevde, 1973) in blood vessels of hypertensive animals. Since intracellular calcium is probably only a small portion of the total tissue calcium (Van Breemen and McNaughton, 1970; Somlyo and Somlyo, 1970), and since smooth muscle cells comprise only a part of the total arterial weight, this increase in Ca^{++} cannot be assigned to any specific sites or cells (Massingham and Shevde, 1973).

Hinke (1965), working with isolated perfused ventral caudal tail arteries from normal and hypertensive animals, found that the muscles from hypertensive arteries exhibited a greater responsiveness to norepinephrine than that from normotensive ones. In another study, Hinke (1966) demonstrated that the dose-response curve relating the amplitude of muscle contraction to calcium ions in the extracellular fluid was shifted to the left in the hypertensive arteries. The shift occurred with both norepinephrine and KCl employed as the excitatory agent. Since calcium is generally believed to be the agent that activates the contractile process in muscle fibers, Hinke suggested that the hyper-responsiveness of diseased arteries may be due either to an improvement in a mechanism of attraction and binding of calcium for subsequent release for contraction or to an improvement in the efficiency of the contractile elements themselves. Somlyo and Somlyo (1970) interpreted Hinke's data by postulating that a hypertensive artery, when stimulated, undergoes a greater than normal increase in membrane permeability to calcium ions or possesses a sluggish relaxing system that does not remove myoplasmic calcium at a normal rate. Therefore, calcium uptake in subcellular organelles may be related in some fashion to changes of vascular reactivity in hypertension. For example, a decreased calcium uptake by subcellular organelles in diseased arteries may increase intracellular free calcium compared to normal arteries; this increase may prolong excitation time and enhance activation of contraction. Alternately, an increased calcium uptake by these organelles may provide a larger calcium pool available for release when stimulated. All these mechanisms conceivably could alter muscular reactivity in hypertension.

(ii) Changes in enzymatic activity

Increased activities of many enzymes have been found using histochemical techniques on tissues or biochemical techniques on homogenates. However, these methods generally lack quantitative accuracy. The following is a partial list: Alkaline phosphatase (Gardner and Laing, 1965; Wyke and Gardner, 1970; Ooshima, 1973), acid phosphatase (Zemplenyi, 1968; Wolinsky et al., 1973; Ooshima, 1973), AMPase, ADPase and ATPase (Oka and Angrist, 1965; Ichijima, 1969). The physiological importance of these enzymes is not clear. They may be related to processes like cell proliferation, ion transport, muscle contraction, etc.

It has been suggested that cyclic AMP, and the enzymes controlling its level in the cell may be responsible for regulating arterial tone and contractility (Triner et al., 1972). Increased cyclic AMP is often associated with relaxation of vascular smooth muscle tone (cf. Baer, 1974). In studies of cyclic AMP level, and of the enzymes controlling its level in blood vessels of various types of hypertensive rats, Amer (1973) and Amer et al. (1974) have found that cyclic AMP content in hypertensive arteries is significantly lower than in normotensive arteries. The change appeared to be due to an increased activity of the cyclic AMP degrading enzyme in hypertensive compared to normotensive arteries, i.e. the cyclic nucleotide phosphodiesterase; there was no difference in activity of the cyclic AMP synthesizing enzyme, adenylate cyclase. Ramanathan and Shibata (1974) have confirmed the result of a lowered cyclic AMP level in arteries of hypertensive rats. However, Triner et al.(1975) have reported that the cyclic AMP level is elevated and that both related enzymes show slightly increased activities in hypertensive arteries. Therefore, the relationship of cyclic AMP level with hypertension is controversial. Besides, the hypothesis of the involvement of cyclic AMP levels in contraction or relaxation of smooth muscle has been questioned (Polacek et al., 1971; Daniel and Crankshaw, 1974; Daniel and Janis, 1975; Ljung et al., 1975). Studies by Andersson (1972) provide evidence of a direct role for cyclic AMP in regulating calcium fluxes (increased calcium binding or transport) in smooth muscle, but Andersson's results have yet to be confirmed (e.g. Batra and Daniel, 1971), and the relationship between cyclic AMP and calcium uptake and release in subcellular fractions of muscle remains controversial.

(iii) Changes in non-collagen protein and collagen biosynthetic activity

Incorporation of ^{14}C-lysine into the non-collagen proteins is increased in the small vessels of SHR (Yamabe and Lovenberg, 1974), and an incorporation of ^{3}H-proline into collagen is increased in the vessels of hypertensive rats (Ooshima et al., 1974, 1975; Gerrity et al., 1975). The above-mentioned results are consistent with Folkow's hypothesis (1956) that increased wall thickness to lumen ratio is the cause of vascular reactivity change in hypertension. However, such studies do not establish this as the unique causative factor for altered vascular reactivity in hypertension. On the other hand, these studies imply altered biochemical operations in arterial smooth muscle in hypertension since this is the site of collagen synthesis (Rose and Klebanoff, 1971). There is the further implicit suggestion of an altered feedback regulation of collagen synthesis in hypertensive arteries.

D) Calcium Utilization in Vascular Tissue

(a) Hypertensive vascular tissue

As previously mentioned Hinke (1965, 1966) suggested that the hyperreactivity of the diseased artery may be due to an abnormailty of calcium metabolism at the cellular or subcellular levels. Finch and Haeusler (1974) reported that in depolarized mesenteric artery preparations the calcium dose-response curves in preparations from normotensive and hypertensive rats were similar. It is known that a high potassium depolarizing medium increases the membrane permeability to extracellular calcium or releases loosely bound calcium from the membrane (Hinke, 1965; Hudgins and Weiss, 1968; Van Breemen, 1969; Van Breemen et al., 1972). However, a more recent study has shown that a high potassium medium may also release some calcium from the intracellular pool (Goodman et al., 1972). Bohr (1974) studied mechanical responses of carotid artery strips from normotensive, DOCA and spontaneously hypertensive rats to different cations (Ba^{++}, Sr^{++}, Mn^{++} and La^{+++}). He found that at the dose ranges from 0.01-1.0 mM the contractile response to Ba^{++} added in the presence of calcium was far less in strips from the hypertensive than from the normotensive rats. On the other hand, contractile responses of strips from the hypertensive rats were greater in response to Sr^{++}, Mn^{++} and La^{+++}, at similar or higher dose ranges. Furthermore, the response of Ba^{++} was found to be highly dependent on the presence of extracellular calcium in preparations from these three types of rats. He suggested that the differences in sensitivity may be related to alterations in the calcium binding properties of the smooth muscle cells of the hypertensive rat. Hudgins and Weiss (1969) showed that barium and strontium can exchange with bound calcium in the rabbit aorta. There appears to be competition between these nonphysiological cations and calcium for membrane binding sites. There is convincing evidence that both manganese (Keene et al., 1972) and lanthanum (Van Breemen et al., 1972) decrease vascular smooth muscle membrane permeability to calcium. These cations probably compete with calcium for membrane sites. Field et al. (1972) suggested that the hypersensitivity to noradrenaline of the aortic smooth muscle of SHR is due to some general alteration of the cell membrane rather than a specific change in the adrenergic α-receptors. If this is true for other vascular smooth muscle of SHR, then the amount of calcium mobilization associated with contraction might be different between SHR and normotensive rats.

From the above results, it is possible that an altered binding and transport system of calcium ion could be part of the cause of the hypertensive state. Since the increase of vascular resistance is determined largely by small blood vessels rather than by large vessels such as aorta, I was specially interested in such vessels (e.g. mesenteric arteries) as a source of subcellular proteins and membranes for studies of calcium uptake. I was also interested in studying aorta and heart in a similar way to see whether any changes apparent in small vessels occur in other components of the cardiovascular system as well.

(b) Normal vascular tissue

The physiological function of calcium uptake by the subcellular components in small vessels is still obscure and therefore information obtained about this will also be valuable. Calcium ion is the agent that activates contractile elements in all types of muscle fibers. However, terminal cisternae, which serve as both a source and a sink for the divalent ions in skeletal (Sandow, 1970) and cardiac muscle (Langer, 1968), have not been found in smooth muscle. Based on evidence obtained from numerous physiological studies performed on various types of smooth muscle fibers, there is currently a consensus that calcium ions associated with mechanical activity in smooth muscle may arise from two types of pools

(Daniel, 1965; Van Breemen and Daniel, 1966; Devine et al., 1973). One is the pool of calcium that is present in the extracellular fluid or that is loosely bound to superficial sites in the muscle fiber: the other is a tightly bound pool of calcium that is sequestered (or located) in the cells. Recent histological and histochemical studies have pointed to three specific loci inside the cell where mobilizable calcium may be sequestered. These are the plasmalemma, the endoplasmic (or sarcoplasmic) reticulum and the mitochondria of the muscle fiber (Somlyo and Somlyo, 1970, 1971; Bohr, 1973).

The contention that an intracellular as well as an extracellular (or superficial) site may store the activator ions needed for contraction does not imply that an intracellular store of calcium plays an equally important physiological role in all types of smooth muscle fibers. For example, the main pulmonary artery of the rabbit, immersed in a calcium-free medium, retains the capacity to contract long after extracellular and presumably loosely-bound calcium ions have been removed (Devine et al., 1972). By contrast the taenia coli of the rabbit, under the same conditions retains its capacity to contract for a much shorter period of time (Devine et al., 1972). Estimates of the amount of endoplasmic reticulum present in the two smooth muscles correlate with these findings. The main pulmonary artery was assessed as having over twice as much endoplasmic reticulum by volume as does the taenia coli (Devine et al., 1972). Devine et al. (1972) estimated the endoplasmic reticulum volume of both rabbit aorta and of guinea pig mesenteric artery; the former was twice as large as the latter. The results suggest that calcium movement from the extracellular or intracellular bound calcium pool during the excitation contraction process should be different between large and small vessels. A smooth muscle that utilizes primarily external or superficially bound calcium for contraction might be expected to have a large part of its capacity to bind or extrude cytoplasmic calcium localized in the plasma membrane. A muscle that mobilizes intracellular calcium might be expected to contain some part of its capacity to actively transport or bind calcium in the membrane of its intracellular organelles (Hurwitz and Suria, 1971; Hurwitz et al., 1973).

E) Aims of this Study

(1) To isolate fractions sufficiently enriched in plasma membrane, endoplasmic reticulum and mitochondria from mesenteric arteries, aortae and ventricles of rats so that the calcium uptake and release by each cell organelle can be studied.

(2) To test the hypothesis that calcium pump(s) exist in the subcellular organelles of vascular smooth muscle (especially on plasma membrane) to control intracellular free calcium concentration.

(3) To test the hypothesis that altered contractile functions of mesenteric arteries, aorta and cardiac muscle of SHR are related to an abnormality of calcium metabolism and certain enzyme activities in subcellular levels.

(4) To explore the relationship between hypertension and the alterations of calcium uptake and certain enzyme activities observed in mesenteric arteries in SHR.

CHAPTER II

ISOLATION AND CHARACTERIZATION OF PLASMA MEMBRANE FROM RAT MESENTERIC ARTERIES

CHAPTER II: ISOLATION AND CHARACTERIZATION OF PLASMA MEMBRANE FROM RAT MESENTERIC ARTERIES

A. Introduction

The isolated mesenteric artery is often used to study changes in vascular reactivity that are associated with a number of types of genetic and experimental hypertension in rats (Armstrong, 1972; Haeusler and Finch, 1972; McGregor and Smirk, 1968). Although procedures are available for the isolation of plasma membrane from a number of tissues (DePierre and Karnovsky, 1973), no method has been reported for the isolation of plasma membrane from small blood vessels. Our laboratory has previously reported a procedure for the isolation of a relatively pure plasma membrane fraction from myometrial smooth muscle (Kidwai et al., 1971a). I have now used a modification of this method to obtain fractions enriched in plasma membrane and endoplasmic reticulum from rat mesenteric arteries. These fractions should be useful for elucidating the role of membranes in various control systems of small blood vessels of normal, experimental and diseased rats.

B. Methods and Materials

Male Wistar rats weighing 400-500 g were killed by a blow on the head, and mesenteric arteries were removed immediately and placed in cold (0-4^{o}C) 0.25 M sucrose prepared in double distilled water. Removal of most of the fat cells was accomplished by stripping of adhering fatty tissue with forceps. The superior mesenteric vein and small veins were also removed and discarded. The resulting tissue was suspended in 0.25 M sucrose and placed in a glass homogenizer with a loose-fitting (0.004 to 0.006 inch clearance) teflon pestle (Tissue Grinder, Potter-Elvehjem, size 23, Kontes). The pestle was rotated at 500 rpm for three strokes in order to remove additional fat cells from the blood vessels. The presence of fat cells was determined by microscopic examination of arteries stained with Sudan black (Carleton and Leach, 1947). The preparation of "mesenteric arteries" included the superior mesenteric, ileal, and jejunal arteries (fig. 1). From twelve rats the total wet weight of fat-free mesenteric arteries ranged from 4.0 to 4.5 g. The arteries obtained by this procedure were homogenized in 0.25 M sucrose with a Polytron PT20 (Kinematica GmbH Luzern-Schweiz) at 15,000 rpm for 15 s. The homogenate was centrifuged at 900 X *g* for 10 min, the pellet was discarded, and the supernatant from a second 900 X *g*/10 min spin was then centrifuged at 104,000 X *g* for 30 min. The sediment was suspended in 1.5 ml of 0.25 M sucrose and layered on a discontinuous sucrose gradient containing 3 ml each of 29%, 37% and 45% sucrose solution (w/w); the concentrations were

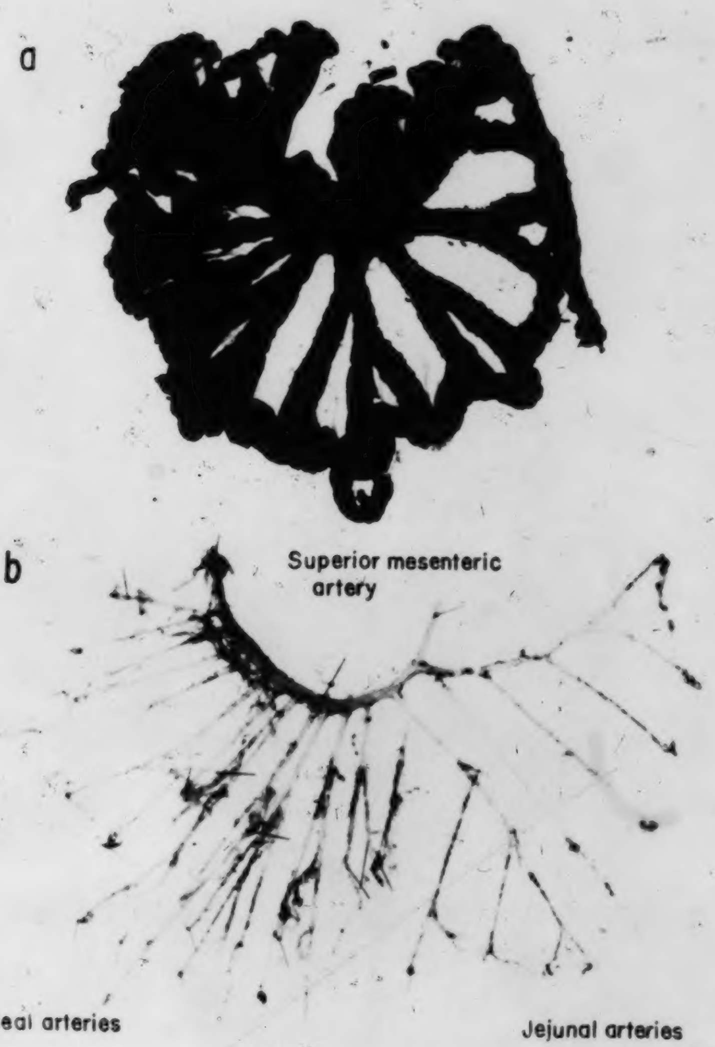
a
b
Superior mesenteric artery
Ileal arteries
Jejunal arteries

Fig. 1. The preparation of "mesenteric arteries" stained with Sudan black: (a) adipose tissue attached; (b) adipose tissue and veins removed. The preparation consists of superior mesenteric, jejunal and ileal arteries.

checked with an Abbe refractometer. The gradient was centrifuged for 120 min at 111,688 X g in rotor SW 41 (Beckman). The preparative scheme is summarized in Figure 2. A band was obtained at each interphase: fraction F_1 between the loading medium and 29% sucrose, F_2 between 29% and 37% sucrose, and F_3 between 37% and 45% sucrose (fig. 3). The fractions were removed by Pasteur pipettes, diluted to a sucrose concentration of 0.25 M, and centrifuged at 104,000 X g for 30 min. The sediment was resuspended in sucrose or buffer as required.

For electron microscopy, tissues or pellets were fixed in 2.5% glutaraldehyde in phosphate buffer (pH 7.1), post-fixed in OsO_4, and embedded in Epon. Sections were stained with uranyl acetate and lead citrate, and photographed on a JEM 7A electron microscope.

5'-Nucleotidase (EC 3.1.3.5) (Song and Bodansky, 1967) and $(Na^+ + K^+)$-ATPase (EC 3.6.1.3) (Kidwai et al., 1973) were determined as previously described; the inorganic phosphate liberated from substrates was determined by the Fiske and SubbaRow (1925) method. The activity inhibited by ouabain (1 mM) was considered to be $(Na^+ + K^+)$-ATPase; this activity was often somewhat less than the increase produced by Na^+ and K^+. Ouabain-sensitive K^+-phosphatase (EC 3.1.3.1) (Kidwai et al., 1971a) alkaline phosphatase (EC 3.1.3.2) (Bessey et al., 1946), and phosphodiesterase I (EC 3.1.4.1) (Touster et al., 1970) were assayed as previously described; p-nitrophenol liberated from substrates was measured at 400 nm. Cytochrome-c oxidase (EC 1.9.3.1) was measured by the method of Cooperstein and Lazarow (1951), and protein by the method of Lowry et al. (1951).

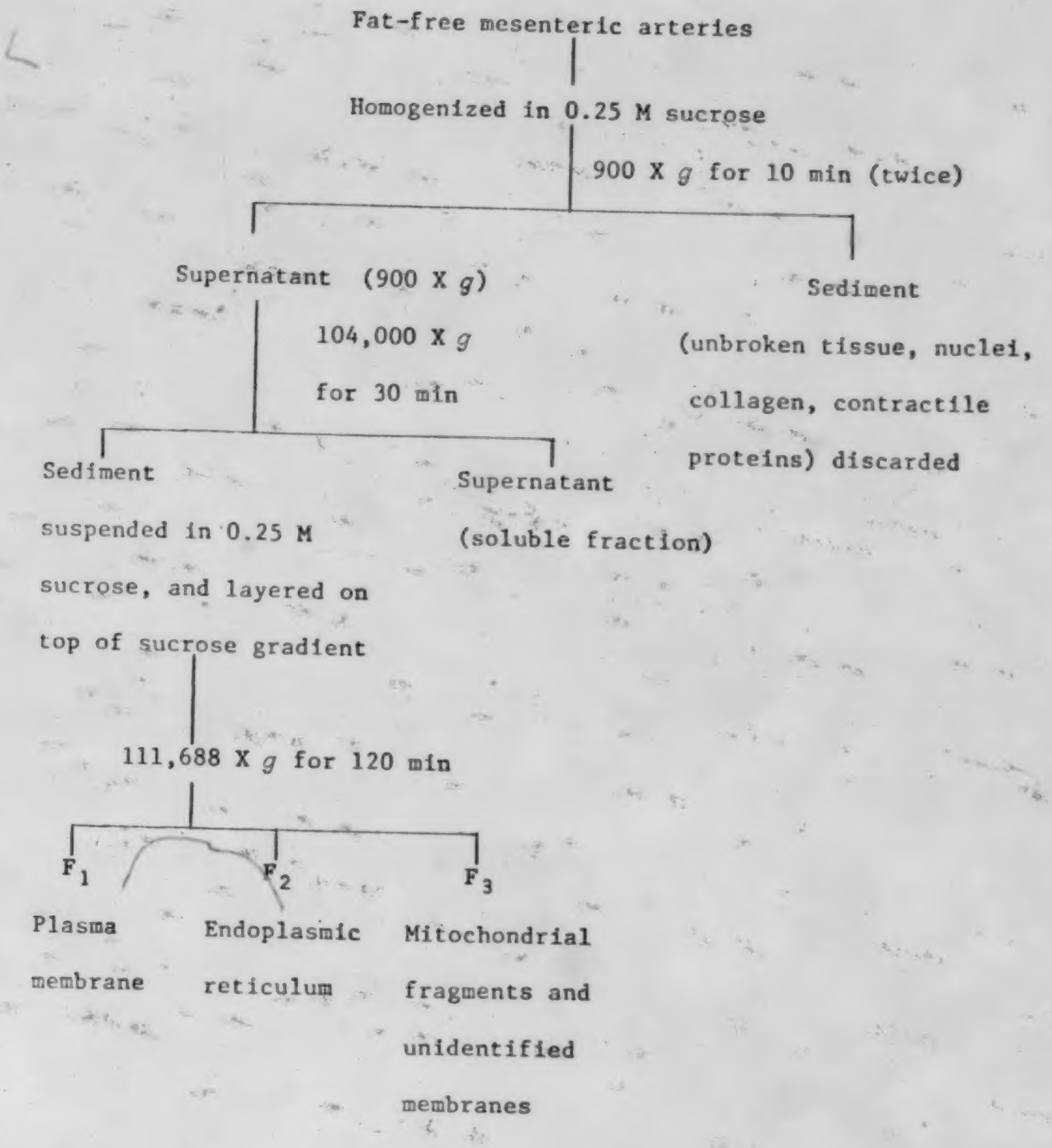
Fat-free mesenteric arteries
Homogenized in 0.25 M sucrose
900 X g for 10 min (twice)
Supernatant (900 X g)
104,000 X g
for 30 min
Sediment
(unbroken tissue, nuclei, collagen, contractile proteins) discarded
Sediment
suspended in 0.25 M sucrose, and layered on top of sucrose gradient
Supernatant
(soluble fraction)
111,688 X g for 120 min
F1
F2
F3
Plasma membrane
Endoplasmic reticulum
Mitochondrial fragments and unidentified membranes

Fig. 2. Procedure for isolation of subcellular fractions.

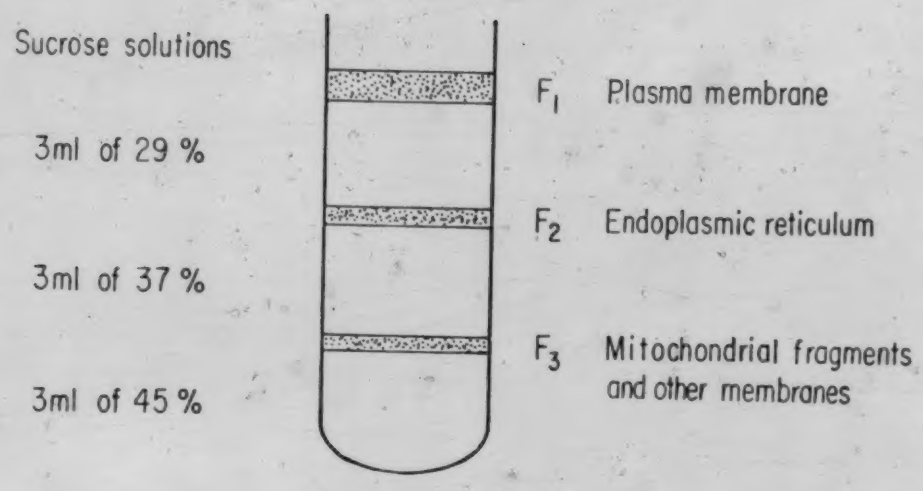
Sucrose solutions
3ml of 29 %
3ml of 37 %
3ml of 45 %
F_1 Plasma membrane
F_2 Endoplasmic reticulum
F_3 Mitochondrial fragments and other membranes

Fig. 3. Schematic representation of the discontinuous sucrose gradient after the 2-hour centrifugation.

Calcium uptake* was studied in reaction mixtures containing a final volume of 1 ml. The composition of the reaction mixture was: 100 mM KCl, 5 mM $MgCl_2$, 0.1 mM $CaCl_2$ labelled with 0.4 μCi/ml $^{45}CaCl_2$, 5 mM ATP, 40 mM imidazole buffer pH 7.0, and 10-40 μg protein. Potassium oxalate (5 mM) and 0.5 mM NaN_3 were added as indicated. The effect of cations and sucrose on calcium uptake was studied by replacing 100 mM KCl with 100 mM NaCl or 200 mM sucrose. The effect of various substrates on calcium uptake was studied by substituting 5 mM of one of the following for ATP: cytidine triphosphate (CTP), guanosine triphosphate (GTP), inosine triphosphate (ITP), uridine triphosphate (UTP), acetyl phosphate (AcP), p-nitrophenyl phosphate (PNPP), adenosine 5'-diphosphate (ADP) or adenosine 5'-monophosphate (AMP). The reaction mixture was incubated at 37°C for 10 min, then 0.8 ml of it was filtered through a millipore filter (25 mm, 0.45 μm, Matheson-Higgins Co.), which had previously been washed with 10 ml of 100 mM KCl, and then with a solution of 10 ml of 8% sucrose and 40 mM imidazole at pH 7.0. Blanks which lacked only subcellular fraction were filtered simultaneously. Filtration took 2-3 s, and was followed by a wash with 10 ml of the same buffered sucrose solution to remove unbound calcium trapped between vesicles. These washes were necessary to give consistent results and low blank values. The amount of calcium uptake by the subcellular fractions was calculated by correcting for the radioactivity in the filter paper without subcellular fraction (blank value). Radioactivity was measured by liquid scintillation counting in a Picker Nuclear Liquimat Model 650. Quenching as measured by the external standards ratio method was negligible, and no correction was necessary.

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* Throughout this thesis calcium uptake is used operationally to describe all processes (binding, transport,etc.) leading to calcium accumulation, and calcium transport refers only to the process whereby calcium is transported actively across the vesicle membrane.

Differential centrifugation was used in an attempt to isolate mitochondria. The homogenate obtained from the Polytron step, as described previously except that the arteries were homogenized twice for 5 s each time, was centrifuged at 900 X *g* for 10 min. The supernatant from a second 900 X *g*/10 min spin was then centrifuged at 13,000 X *g* for 30 min. The pellet was suspended in 0.25 M sucrose and centrifuged at 900 X *g* for 10 min; then the supernatant obtained was centrifuged at 13,000 X *g* for 30 min. This pellet was suspended in 0.25 M sucrose or buffer, which was the mitochondrial enriched fraction.

Solutions were prepared from distilled-deionized water and analytical grade reagents. Organic compounds were the highest purity available from Sigma; $^{45}CaCl_2$ was obtained from Amersham-Searle.

Values were compared by the Student t-test, and differences with $P < 0.05$ were considered significant. Standard errors of the means are bracketed in the figures.

C. Results and Discussion

The superior mesenteric, jejunal and ileal arteries were included in this preparation. Contamination of the artery preparation by fat cells was low, based on examination of preparations stained with Sudan black (fig. 1b); for comparison, mesenteric arteries from which adipose tissue was not removed, are included (fig. 1a). A typical electron micrograph of smooth muscle cells of rat mesenteric artery is shown in figure 4a; little endoplasmic reticulum and few mitochondria are present. After subcellular fractions were isolated, both enzymatic marker and electron microscopic techniques were used to characterize them.

The plasma membrane (PM = F_1) fraction obtained from the top of the gradient was vesicular when examined in the electron microscope, and the vesicle membrane was seen (in properly oriented sections) to show the typical bilayer structure (figs. 4b, 5a). The PM fraction made up 5.4% of the total protein of the 900 X *g* supernatant (Table 1). 5'-Nucleotidase, ouabain-sensitive (Na^++K^+)-ATPase, phosphodiesterase I, alkaline phosphatase, and ouabain-sensitive K^+-phosphatase (Table 2), which are widely used as plasma membrane markers (DePierre and Karnovsky, 1973; Touster et al., 1970), were found to be enriched in this fraction. The specific activities were 4 to 6 times higher than those in other subcellular fractions.

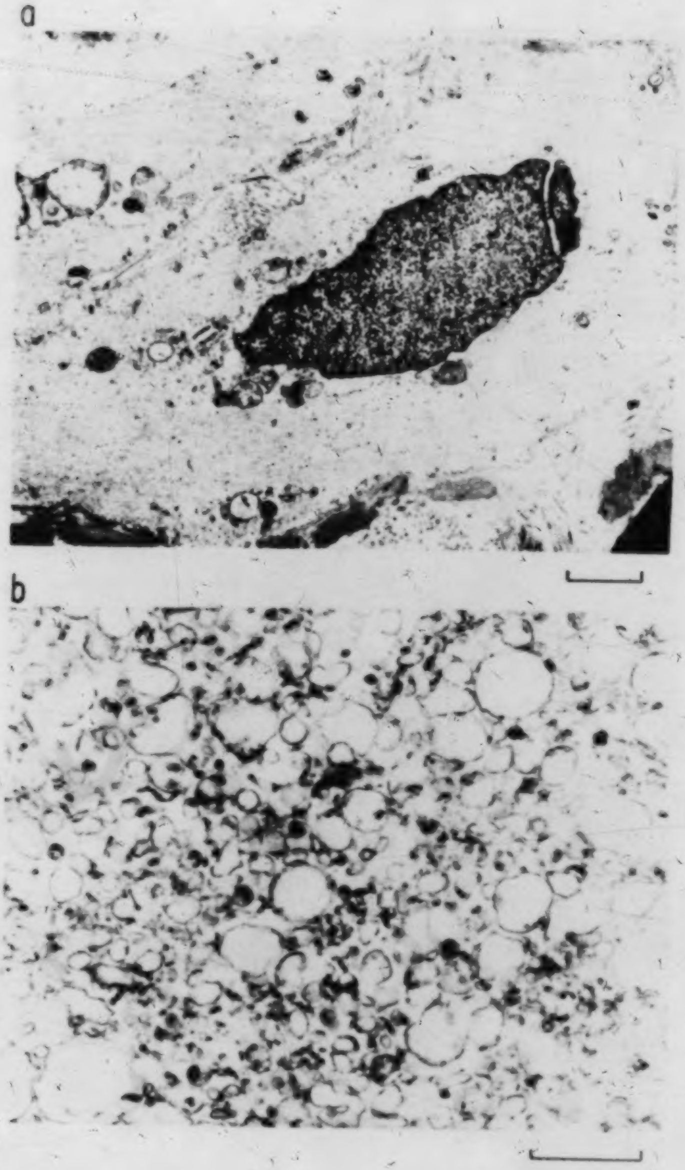

Fig. 4. Electron micrographs of vascular smooth muscle cells of rat mesenteric artery and isolated plasma membrane enriched fraction. (a) Vascular smooth muscle of rat mesenteric artery; (b) fraction F_1 (plasma membrane). Each bar represents one micron.

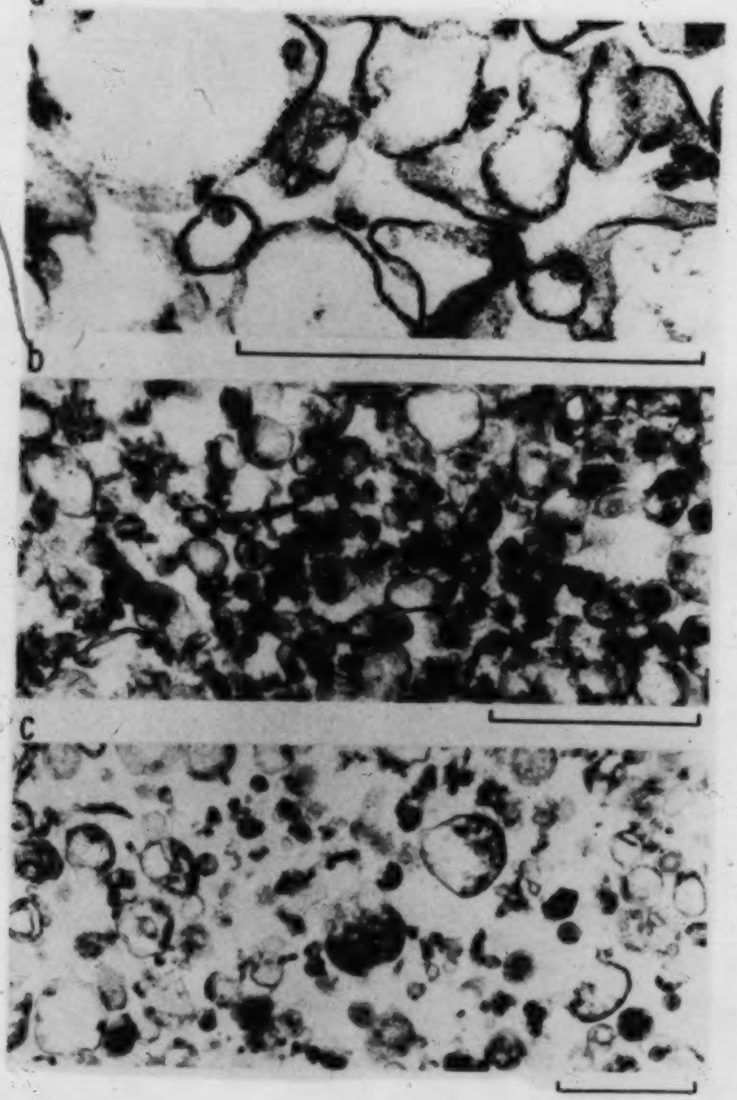

Fig. 5. Electron micrographs of various fractions isolated after density gradient centrifugation. (a) Fraction F_1 (plasma membrane) at higher magnification; (b) fraction F_2 (endoplasmic reticulum); (c) fraction F_3 (mitochondrial fragments and other membranes). Each bar represents one micron.

Table 1. Protein distribution in subcellular fractions from rat mesenteric arteries

Fraction	Total Protein Value (mg)	% of 900 X *g* Supernatant
900 X *g* Supernatant	34.1 ± 1.38	100
Soluble	29.6 ± 1.12	86.8 ± 3.3
F_1 (PM)	1.85 ± 0.096	5.4 ± 0.3
F_2 (ER)	0.460 ± 0.037	1.4 ± 0.1
F_3	0.498 ± 0.044	1.5 ± 0.2

N is the number of experiments, mesenteric arteries from 12 rats in one experiment, performed on separate preparations. N = 4.

PM = plasma membrane enriched fraction; ER = endoplasmic reticulum enriched fraction.

Table 2. Specific activities of 5'-nucleotidase, ouabain-sensitive (Na^++K^+)-ATPase, phosphodiesterase I, alkaline phosphatase, ouabain-sensitive K^+-phosphatase, and cytochrome-c oxidase in subcellular fractions from rat mesenteric arteries

Enzyme	F_1 (PM)	F_2 (ER)	F_3	N
5'-nucleotidase	58.4 ± 3.9	9.5 ± 1.1	9.4 ± 0.9	7
ouabain-sensitive (Na^++K^+)-ATPase	15.1 ± 3.1	4.1 ± 0.7	3.9 ± 1.0	5
phosphodiesterase I	21.81 ± 0.82	5.06 ± 0.45	4.72 ± 0.40	5
alkaline phosphatase	7.66 ± 0.32	2.14 ± 0.29	1.85 ± 0.21	5
ouabain-sensitive K^+-phosphatase	2.5 ± 0.2	0.6 ± 0.1	0.3 ± 0.1	5
cytochrome-c oxidase	0.57 ± 0.2	1.47 ± 0.3	3.84 ± 0.5	6

5'-Nucleotidase and ouabain (1mM)-sensitive (Na^++K^+)-ATPase activities are expressed as μmoles Pi released mg^{-1} protein h^{-1}. Phosphodiesterase I, alkaline phosphatase and ouabain-sensitive K^+-phosphatase activities are expressed as μmoles p-nitrophenol released mg^{-1} protein h^{-1}. Cytochrome-c oxidase activity is expressed as reduction in optical density at 550 nm mg^{-1} protein min^{-1}. N is the number of experiments performed on separate preparations.

The endoplasmic reticulum (ER = F_2) fraction obtained from the middle layer of the gradient has a vesicular appearance in the electron microscope; vesicles in this fraction were not as homogeneous in size and shape as those of the PM fraction (fig. 5b). I was unable to determine the extent of cross-contamination of plasma membrane by endoplasmic reticulum by using enzyme activities because there are no well-established marker enzymes for endoplasmic reticulum of smooth muscle. However, oxalate was reported to stimulate calcium uptake by skeletal muscle plasma membrane vesicles much less than that of sarcoplasmic reticulum (Sulakhe et al., 1973). Hence, oxalate stimulation of calcium uptake may be characteristic of endoplasmic reticulum. I found that ATP-dependent calcium uptake in the presence of oxalate was increased only 6.6% in the PM fraction, but 204.5% in the ER fraction, and 47.5% in the F_3 fraction (Table 3). If oxalate stimulation of calcium uptake is unique to endoplasmic reticulum, then there is only a minor contamination of plasma membrane by endoplasmic reticulum. My electron micrographs (for example, fig. 4a) revealed a minimal quantity of endoplasmic reticulum in mesenteric arteries. This has been well-documented by Devine et al. (1972) for guinea pig mesenteric artery, where endoplasmic reticulum occupied only 1.8% of the cell volume. This small volume compared to skeletal and cardiac muscle is consistent with the small amount of protein found in this fraction relative to the PM fraction (Table 1); however, the per cent yield of these fractions is not known.

Table 3. Effect of potassium oxalate on ATP-dependent calcium uptake by subcellular fractions from rat mesenteric arteries

Fraction	No ATP	5 mM ATP	5 mM ATP + 5 mM oxalate	% increase in ATP-dependent calcium uptake by oxalate
F_1 (PM)	5.16 ± 0.20	15.32 ± 0.32	16.04 ± 0.72	6.6 ± 6.1
F_2 (ER)	2.75 ± 0.20	6.92 ± 0.81	14.63 ± 0.56	204.5 ± 41.3
F_3	1.46 ± 0.01	7.21 ± 1.03	9.31 ± 1.40	47.5 ± 26.5

Values are expressed as μmoles calcium accumulated g^{-1} protein in 10 min. N = 4.

Note: In the absence of ATP and in the presence of 5 mM oxalate, the values of calcium uptake by the subcellular fractions were similar to those values in the "No ATP" column.

F_3 contained few if any intact mitochondria (fig. 5c), although it exhibited the highest cytochrome-c oxidase activity of the subcellular fractions isolated (Table 2). However, NaN_3 (0.5 mM) did not inhibit the calcium uptake by this fraction (Table 4), and the magnitude of calcium uptake by this fraction was very low compared to that expected (Batra and Bengtsson, 1972). Therefore, a differential centrifugation method was used to isolate a mitochondrial enriched fraction. It contained 3.2 ± 0.2% of the total protein of the 900 X *g* supernatant (23.5 ± 1.6 mg obtained from 12 rats). The amount of calcium uptake in the presence of 17 µM free calcium and 5 mM ATP by this fraction was 55 ± 3 µmoles calcium/g protein/10 min, 52 ± 2% of which was sensitive to azide (n = 4). It is likely that this fraction was highly contaminated by other subcellular components.

Since the subcellular fractions isolated from rat mesenteric arteries exhibit ATP-dependent calcium uptake, it was necessary to characterize this process in more detail. The effect of cations and sucrose on calcium uptake by both PM and ER fractions is shown in Table 5. In the presence of Mg^{2+} and ATP, the calcium uptake by both PM and ER fractions was relatively unaffected by the presence of 100 mM KCl or NaCl. However, in the presence of ATP, calcium uptake by the PM fraction was slightly and significantly decreased by 200 mM sucrose. The effect of various substrates on calcium uptake was also studied. Calcium uptake by the PM or ER fraction was enhanced by ATP, but not by CTP, GTP, ITP, UTP, AcP, or PNPP (Table 6). This property of calcium uptake appears to be

Table 4. Effect of sodium azide on calcium uptake in the presence of ATP by subcellular fractions from rat mesenteric arteries

Fraction	5 mM ATP	5 mM ATP + 0.5 mM NaN_3	% change
F_1 (PM)	15.47 ± 0.39	15.87 ± 0.77	+ 2.65 ± 2.52
F_2 (ER)	7.86 ± 0.16	7.45 ± 0.33	- 5.21 ± 3.43
F_3	8.63 ± 1.16	7.50 ± 1.08	- 12.97 ± 4.63

Values are expressed as μmoles calcium accumulated g^{-1} protein in 10 min.

N = 4

Table 5. Effect of cations and sucrose on calcium uptake by plasma membrane and endoplasmic reticulum fractions from rat mesenteric arteries

Fraction	KCl 100 mM	NaCl 100 mM	Sucrose 200 mM	Without KCl
F_1 (PM)	100	98.6 ± 5.6	83.2 ± 4.7*	98.0 ± 5.9
F_2 (ER)	100	104.1 ± 3.2	96.3 ± 7.5	101.2 ± 3.1

Values are expressed as mean ± S.E. of % of calcium uptake in control solution, which contained 100 mM KCl, 5 mM ATP, 5 mM $MgCl_2$, 0.1 mM $CaCl_2$ labelled with 0.4 µCi/ml $^{45}CaCl_2$, 40 mM imidazole buffer pH 7.0, and 10-40 µg/ml protein, and which yielded 17 µM free calcium. Incubation time was 10 min. N = 4.

* Denotes values significantly different from 100%.

Table 6. Calcium uptake by plasma membrane and endoplasmic reticulum fractions from rat mesenteric arteries in the presence of various substrates

Substrate (5 mM)	FRACTION	
	PM	ER
ATP	100	100
GTP	46.1 ± 1.8	52.6 ± 2.0
ITP	42.8 ± 3.9	44.3 ± 3.6
CTP	40.9 ± 2.6	37.4 ± 1.8
UTP	51.1 ± 2.6	47.8 ± 4.9
AcP	54.6 ± 2.3	52.7 ± 4.1
PNPP	45.4 ± 6.2	40.1 ± 5.5
ADP	42.6 ± 3.0	53.3 ± 7.8
AMP	51.2 ± 3.3	54.8 ± 6.0
None	43.5 ± 5.2	45.8 ± 7.6

Values are expressed as mean ± S.E. of % calcium uptake in the presence of 5 mM ATP, 17 μM free calcium.

Incubation time was 10 min.

N = 4.

different from that of skeletal muscle (De Meis, 1969; Inesi, 1971; Martonosi and Feretos, 1964), but similar to that of myometrial smooth muscle (Janis, Crankshaw and Daniel, to be published). The amount of ATP-dependent calcium uptake by the PM fraction was about two times that by the ER fraction. However, in the presence of oxalate, the amount of calcium uptake by each fraction was similar in a 10 min incubation (Table 3). These results suggest that the calcium accumulation properties of plasma membrane may be different from those of endoplasmic reticulum, perhaps because of differences in the passive permeability of the vesicles to calcium, or to differences in the permeability of the vesicles to oxalate.

This method of isolating fractions from rat mesenteric arteries does not require exhaustive washings with a concentrated salt solution, and it provides a reasonable yield of plasma membrane and endoplasmic reticulum in less than 6 hours. All plasma membrane marker enzymes studied were concentrated 4 to 6 times in the PM fraction compared to the ER fraction. This suggested that the ER fraction contained a maximum of 25% plasma membrane. The PM fraction exhibited little increase in calcium uptake by the addition of oxalate in contrast to the ER fraction. If this is a valid "marker" for endoplasmic reticulum, then the contamination of the PM fraction by the ER fraction may be very small. Mitochondrial contamination of the PM fraction was inconsequential as measured by the azide sensitivity of calcium uptake. The F_3 fraction obviously contained mostly non-mitochondrial membrane material which accounted for calcium uptake. Its calcium uptake was relatively insensitive to oxalate. The plasma membrane and endoplasmic reticulum fractions isolated in this study will be useful for studying hormone and drug binding, calcium transport, and a variety of other biochemical and pharmacological parameters relating to small vessels of normal and abnormal animals.

D. Summary

A method for isolating a plasma membrane enriched fraction and also other subcellular fractions from rat mesenteric arteries by the use of a discontinuous sucrose density gradient is described. Electron microscopy showed both plasma membrane and endoplasmic reticulum fractions to be composed of vesicles. 5'-Nucleotidase, alkaline phosphatase, ouabain-sensitive $(Na^{+}+K^{+})$-ATPase and K^{+}-phosphatase, and phosphodiesterase I were concentrated in the plasma membrane fraction. The increase in ATP-dependent calcium uptake in the presence of oxalate was greater in the endoplasmic reticulum than in the plasma membrane fraction. The lack of inhibition of active calcium uptake by azide suggests that the plasma membrane enriched fraction was relatively free of mitochondrial contamination. Calcium uptake by the plasma membrane or the endoplasmic reticulum fraction was not enhanced by high-energy compounds other than ATP, and was little affected by 100 mM KCl or NaCl in the Mg^{++}-containing medium. Subcellular fractions isolated by this method will be useful for investigating the biochemistry of small blood vessels of the rat.

CHAPTER III

CALCIUM UPTAKE AND ENZYMATIC ACTIVITIES OF SUBCELLULAR FRACTIONS FROM MESENTERIC ARTERIES OF 5-6 MONTHS OLD SPONTANEOUSLY HYPERTENSIVE RATS

CHAPTER III: CALCIUM UPTAKE AND ENZYMATIC ACTIVITIES OF SUBCELLULAR FRACTIONS FROM MESENTERIC ARTERIES OF 5-6 MONTHS OLD SPONTANEOUSLY HYPERTENSIVE RATS.

A. Introduction

Small resistance vessels play the major role in determining peripheral vascular resistance, and they are important sites of decreased conductance in essential hypertension (Freis, 1960; Page and McCubbin, 1966). This increase in resistance may result from supersensitivity to vasoconstrictor stimuli or from altered vascular structure (Somlyo and Somlyo, 1970). Many reports have shown increased vascular reactivity in hypertensive arteries *in vivo* (Carlini et al., 1959; Lais et al., 1974; Weiss, 1974) and *in vitro* (Collis and Alps, 1975; Haeusler and Finch, 1972; McGregor and Smirk, 1970). In order to understand the mechanism of this vascular reactivity change, it is essential to analyze each step of the series of events which link the application of a vasoactive stimulant to the hemodynamic response. According to Johansson (1974), the delivery of calcium and ATP (adenosine triphosphate) to contractile proteins are the most susceptible steps to alteration, and hence their dysfunction could underlie altered vascular reactivity. Calcium is believed to be the rate-limiting determinant of vasoconstriction; therefore a derangement in its function and utilization is a plausible cause of vascular hyper-reactivity.

After having isolated fractions enriched in plasma membrane and endoplasmic reticulum from mesenteric arteries of normotensive Wistar rats (NWR) (see Chapter II), I applied this technique to a comparative study of the calcium uptake properties and enzymatic activities of these membranes from SHR with those from normotensive rats.

Several investigators have observed that enzyme activities such as AMPase (adenosine monophosphatase) and alkaline phosphatase are increased in arteries of hypertensive animals, and they have suggested that these alterations might be involved in reactivity change in hypertension (Gardner and Laing, 1965; Ichijima, 1969; Oka and Angrist, 1965; Ooshima, 1973). I have examined whether such changes are specific for certain phosphatases, or whether the activities of several phosphatases are increased. In addition, other enzymes which are believed to be located mainly in the plasma membrane and are involved in ion transport, RNA metabolism, and cell proliferation were studied.

Folkow et al. (1958) have proposed that a structural change leading to an increased wall thickness to lumen size ratio may be the underlying cause of increased vascular reactivity in essential hypertension. I hoped to determine by my studies whether or not alterations in biochemical parameters were present: the presence of changes in enzyme activities associated with cell proliferation and the lack of alterations in calcium uptake properties would support Folkow's hypothesis. Several pharmacological probes were used to characterize these membranes, including both calcium or divalent ion ionophores (X-537A, A23187), and calcium "antagonists" (SKF-525A, diazoxide and propranolol). Both propranolol and diazoxide are antihypertensive agents; diazoxide is of particular interest because it has been reported to be a more potent calcium antagonist in hypertensive than in normotensive vessels (Janis and Triggle, 1973; Wohl et al., 1968 .

B. Methods and Materials

Male spontaneously hypertensive Wistar rats of the Okamoto and Aoki (1963) strain and normotensive Wistar rats were 5 to 6 months of age and received a standard diet and tap water. Systolic blood pressure was recorded from the tail of prewarmed unanesthetized rats with a pneumatic pulse transducer, an electrosphygmograph and a physiograph Four-A (E & M Instrument Company, Houston, Texas). The average of the three lowest of five readings obtained was recorded as the blood pressure of each rat. For a single set of experiments, 6 to 12 rats of SHR and NWR were killed by a blow on the head. The mesenteric arteries were removed immediately and placed in cold (0-4^{o}C) 0.25 M sucrose prepared in double-distilled water. The wet weight of fat-free mesenteric arteries ranged from 4.0 to 4.5 g from 12 NWR, and from 3.1 to 3.6 g from 12 SHR. The subcellular fractions enriched in plasma membrane, endoplasmic reticulum, or mitochondrial and other membranes were isolated and studied by electron microscopic, enzymatic and calcium uptake techniques as previously described (see Chapter II).

Leucine aminopeptidase (EC 3.4.1.1) was assayed by the method of Goldbarg and Rutenburg (1958). Mg^{++}-ATPase (Mg^{++}-adenosine triphosphatase) and (Ca^{++}+Mg^{++})-ATPase were assayed as follows: the reaction mixture contained 100 mM KCl, 5 mM Mg-ATP, 20-40 μg membrane protein, 40 mM imidazole buffer pH 7.0, with 0.1 mM $CaCl_2$ for (Ca^{++}+Mg^{++})-ATPase activity, or without $CaCl_2$ for Mg^{++}-ATPase, in a total volume of 1 ml.

In some cases, suitable amounts of EGTA (ethyleneglycol-bis-(β-amino ethyl ether)-N,N'-tetraacetic acid) were added to produce a free calcium concentration of 0.3 μM and 1 μM. The reaction mixtures were preincubated at 37°C for 5 min, and then incubated for 10 min after the reaction was started by adding a subcellular membrane fraction. The reaction was stopped by the addition of 1 ml of cold 10% TCA (trichloroacetic acid). Inorganic phosphate liberated was measured by the method of Fiske and SubbaRow (1925).

Active calcium uptake (that dependent on the presence of ATP) was carried out in reaction mixtures containing 100 mM KCl, 5 mM $MgCl_2$ 5 mM ATP (disodium or magnesium salt), 0.1 mM $CaCl_2$ labelled with 0.4 μCi $^{45}CaCl_2$, 20-40 μg membrane protein, 40 mM imidazole buffer at pH 7.0 in a total volume of 1 ml. In some experiments of active calcium uptake, suitable amounts of EGTA were used to adjust the free calcium concentrations to 0.3 and 1 μM. The reaction mixture yielded a free calcium concentration of 17 μM without EGTA. I used the method of Katz et al. (1970) and the binding constants of Godt (1974) to calculate free calcium levels; I considered pH, and the concentrations of ATP, EGTA, calcium ion, magnesium ion, and buffer in these calculations.

Passive calcium uptake (in the absence of ATP) was studied in a reaction mixture that contained, in a total volume of 1 ml: 100 mM KCl, 5 mM $MgCl_2$, 0.1 mM $CaCl_2$ labelled with 0.8 μCi $^{45}CaCl_2$, 30-60 μg membrane protein, 40 mM imidazole buffer at pH 7.0. Concentrations of 0.3 and 1 μM free calcium were obtained by adding appropriate amounts of EGTA. To obtain higher calcium concentrations, additional $CaCl_2$ was added.

The active and passive calcium uptake by the membrane fractions was studied by the use of the millipore filtration technique (see Chapter II).

X-537A (Hoffman-LaRoche), A23187 (Eli Lilly), and SKF-525A (Smith Kline and French Labs) were dissolved in 95% ethanol; 10 µl of each drug was added to a total of 1 ml of reaction mixture. The other reagents and drugs were dissolved in 40 mM imidazole buffer at pH 7.0; except diazoxide which required a pH of 7.2. Solutions were prepared from distilled deionized water and analytical grade reagents. Organic compounds were the highest purity available from Sigma; $^{45}CaCl_2$ was obtained from Amersham-Searle.

Student's t-test was used to test for significance. Differences of $P < 0.05$ were considered significant. All values are expressed in the Results as mean ± standard error.

C. Results

a) Comparisions of enzyme activities in fractions from NWR and SHR arteries.

The blood pressures of SHR (191 ± 7.0 mm Hg) were significantly greater than those of NWR (125 ± 5.5 mm Hg) at 5-6 months of age, while the body weights of SHR (356 ± 5 g) were significantly less than those of NWR (482 ± 7 g) (N = 40). Similar results have been reported previously for SHR of this age (Field et al., 1972).

The subcellular fractions of NWR and SHR arteries were isolated on the discontinuous sucrose density gradient as previously described (see Chapter II); these fractions were designated: plasma membrane, F_1 (PM) (the loading medium and 29% sucrose interphase); endoplasmic reticulum, F_2 (ER) (29%/37% interphase); and F_3 (37%/45% interphase). The protein distribution in the subcellular fractions of NWR and SHR arteries from discontinuous sucrose density gradient is shown in Table 1. Generally, the total amount of protein in the subcellular fractions from SHR arteries was 20% less than that from NWR arteries. However, when the results are expressed as per cent of the total protein in the supernatant from the 900 X *g* centrifugation, the distribution of protein in the various fractions from the two types of rat are similar. Electron micrographs of membrane fractions from NWR arteries revealed that these fractions were similar to those of SHR (see Chapter II).

The specific activities of plasma membrane marker enzymes:

Table 1. Protein distribution in subcellular fractions from NWR and SHR mesenteric arteries

	NWR		SHR	
Fraction	Total protein (mg)	% of 900 X *g* supernatant	Total protein (mg)	% of 900 X *g* supernatant
900 X *g* supernatant	34.1 ± 1.38	100	27.6 ± 1.32	100
Soluble	29.6 ± 1.12	86.8 ± 3.3	24.2 ± 1.07	87.6 ± 3.9
F_1 (PM)	1.85 ± 0.096	5.4 ± 0.3	1.37 ± 0.063	5.0 ± 0.2
F_2 (ER)	0.460 ± 0.037	1.4 ± 0.1	0.339 ± 0.033	1.2 ± 0.1
F_3	0.498 ± 0.044	1.5 ± 0.2	0.349 ± 0.034	1.3 ± 0.1

N is the number of experiments; in each experiment 12 NWR and 12 SHR were used. N = 4

PM = plasma membrane-enriched fraction; ER = endoplasmic reticulum-enriched fraction.

ouabain-sensitive K^+-activated phosphatase (Table 2), 5'-nucleotidase, leucine aminopeptidase and phosphodiesterase I (Table 3) were significantly greater in S_1 (PM fraction from SHR arteries) than in N_1 (PM fraction from NWR arteries), but not in the other subcellular fractions of NWR and SHR arteries. Alkaline phosphatase and Mg^{++}-ATPase (Table 3) were significantly greater in all the subcellular fractions from SHR compared with those from NWR. [illegible] all cases (Ca^{++}+ Mg^{++})-ATPase was not significantly different from that of Mg^{++}-ATPase, even in the presence of EGTA.

b) Comparisons of calcium uptake activity of
fractions from NWR and SHR arteries.

(1) Active calcium uptake. At 17 μM free calcium (fig. 1), uptake by S_1 was significantly greater than that by N_1 (19.99 ± 0.90 vs. 15.01 ± 1.15 μmoles calcium/g protein/10 min). The time course of active calcium uptake by N_1 and S_1 at 17 μM free calcium concentration is shown in fig. 2. Active calcium uptake by S_1 was greater than that of N_1 at 3, 10 and 20 min incubation times. Passive calcium uptake by PM fractions of SHR was not different from that of NWR at this free calcium concentration, and uptake by both preparations reached a maximum at 1 to 3 min.

(2) Effect of oxalate on active calcium uptake. In the presence of oxalate, active calcium uptake by the PM fraction from SHR remains significantly greater than that from NWR (Table 4), as expected because oxalate does not affect active calcium uptake by either PM

Table 2. Specific activities of ouabain-sensitive K^+-activated phosphatase in subcellular fractions from NWR and SHR mesenteric arteries

Fraction	NWR		SHR	
	K^+-stimulated	Sensitive to ouabain (10^{-3} M)	K^+-stimulated	Sensitive to ouabain (10^{-3} M)
F (PM)	3.5 ± 0.4	2.4 ± 0.2	*6.4 ± 0.2	*3.9 ± 0.5
F (ER)	1.1 ± 0.2	0.5 ± 0.1	1.5 ± 0.2	0.9 ± 0.3
F	0.9 ± 0.3	0.3 ± 0.1	1.1 ± 0.3	0.5 ± 0.1

Values are expressed as μmoles p-nitrophenol released mg^{-1} protein h^{-1}.

N = 4

* Denotes values significantly different from values obtained from the corresponding fraction of NWR arteries.

Table 3. Specific activities of 5'-nucleotidase, leucine aminopeptidase, phosphodiesterase I, alkaline phosphatase and Mg^{++}-ATPase in subcellular fractions from NWR and SHR mesenteric arteries

Enzyme and type of rat	Fraction		
	F_1 (PM)	F_2 (ER)	F_3
5'-Nucleotidase			
Normotensive	58.35 ± 3.90	9.52 ± 1.10	9.45 ± 0.90
Hypertensive	*78.90 ± 5.70	11.95 ± 0.90	11.66 ± 1.60
Leucine aminopeptidase			
Normotensive	2.80 ± 0.16	1.07 ± 0.09	0.89 ± 0.05
Hypertensive	*3.65 ± 0.13	1.21 ± 0.08	0.97 ± 0.05
Phosphodiesterase I			
Normotensive	21.81 ± 0.82	5.06 ± 0.45	4.72 ± 0.40
Hypertensive	*25.62 ± 0.72	6.50 ± 0.69	5.48 ± 0.66
Alkaline phosphatase			
Normotensive	7.66 ± 0.32	2.14 ± 0.29	1.85 ± 0.21
Hypertensive	*11.64 ± 1.11	*3.17 ± 0.38	*3.35 ± 0.41
Mg^{++}-ATPase			
Normotensive	361 ± 15.9	147 ± 10.5	105 ± 7.79
Hypertensive	*620 ± 9.17	*194 ± 12.1	*172 ± 8.34

Specific activities are expressed as µmoles phosphate released mg^{-1} protein h^{-1} for 5'-nucleotidase, and Mg^{++}-ATPase, and as µmoles β-naphthylamine released mg^{-1} protein h^{-1} for leucine aminopeptidase, and as µmoles p-nitrophenol released mg^{-1} protein h^{-1} for phosphodiesterase I and alkaline phosphatase.

* Denotes values significantly different from normotensive.

N = 5 except for 5'-nucleotidase where N = 7.

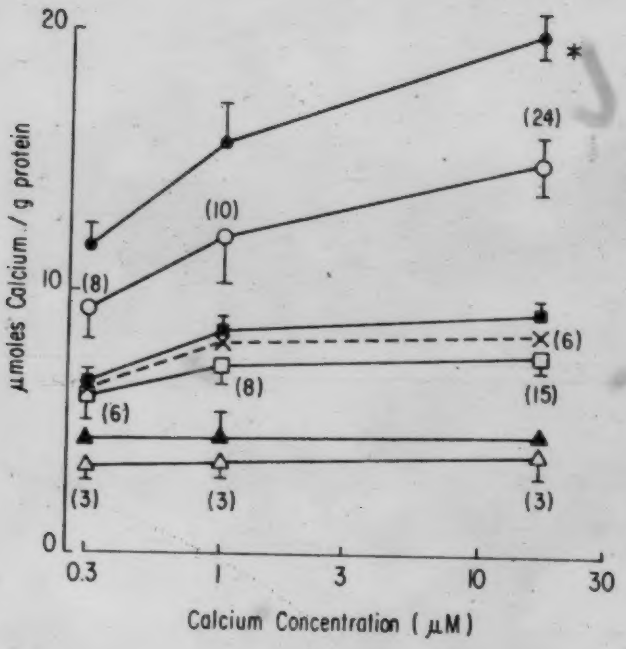

Fig. 1. Effect of calcium concentration on active calcium uptake by subcellular fractions from mesenteric arteries of NWR (open symbols) and SHR (closed symbols): circles, plasma membrane; squares, endoplasmic reticulum; triangles, 900 X *g* supernatant; cross markers, F_3. The values for F_3 from NWR arteries were not significantly different from those from SHR. The length of vertical bar to the symbol represents one standard error of the mean. Numbers in the parentheses are the numbers of experimental sets performed. Asterisk indicates a significant difference from normotensive values. Incubation time was 10 min.

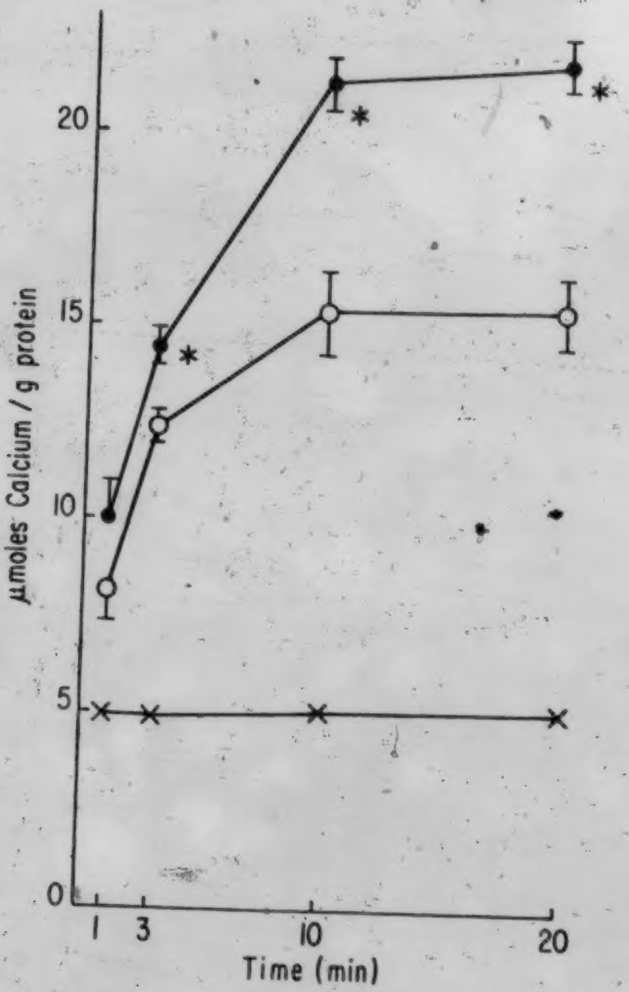

Fig. 2. Time course of calcium uptake by plasma membrane fractions from mesenteric arteries of NWR (open circles) and SHR (closed circles) in the presence of 5 mM ATP. Calcium uptake in the absence of ATP for this fraction from NWR arteries is also shown (x). The ATP-independent values for SHR (not shown) were not significantly different from those for NWR. Asterisk indicates a significant difference from normotensive value. N = 5.

Table 4. Effect of ATP and potassium oxalate on calcium uptake by subcellular fractions from NWR and SHR mesenteric arteries

Fraction	No ATP	5 mM ATP	5 mM oxalate + 5 mM ATP	N
N_1 (PM)	5.23 ± 0.48	15.32 ± 0.32	16.04 ± 0.72	4
S_1 (PM)	4.88 ± 0.58	*22.18 ± 0.44	*23.70 ± 0.75	4
N_2 (ER)	2.74 ± 0.20	7.72 ± 0.96	†13.94 ± 2.36	8
S_2 (ER)	2.75 ± 0.26	7.91 ± 0.78	†15.00 ± 2.43	8
N_3	1.46 ± 0.20	8.13 ± 1.22	8.87 ± 1.06	5
S_3	1.65 ± 0.22	8.54 ± 1.06	9.60 ± 1.66	6

Values are expressed as μmoles calcium taken up g^{-1} protein in 10 min.

* Denotes values significantly different from those obtained for the corresponding fractions from NWR arteries.

† Denotes values significantly different from those obtained in the absence of oxalate of the same fractions of either NWR or SHR arteries.

fraction (see Chapter II). Although oxalate does enhance active calcium uptake by the ER fraction, this increase was similar for this fraction from NWR and SHR. Active calcium uptake by F_3 was not increased significantly by the addition of oxalate.

(3) Effect of azide on active calcium uptake. In the presence of azide, active calcium uptake was again significantly greater for S_1 than for N_1 (Table 5). The calcium uptake of the other subcellular fractions from NWR and SHR was not significantly different in the presence of azide. The lack of effect of azide on active calcium uptake demonstrates the absence of significant contamination of any of the fractions by inner mitochondrial membrane.

(4) Effect of ionophores on active calcium uptake. Ionophores partially inhibited active calcium uptake by all the subcellular fractions of NWR and SHR arteries; the vehicle for the ionophores, 95% ethanol, had no effect itself (Table 6). In the presence of ionophores, calcium uptake by the subcellular fractions from SHR arteries was decreased more than for those from NWR arteries. The residual calcium uptake by the subcellular fractions from NWR and SHR arteries was not significantly different in the presence of ionophores.

(5) Comparison of passive calcium uptake by the PM fractions from NWR and SHR arteries. Passive calcium uptake by the PM fraction from NWR arteries at 1 mM $CaCl_2$ in the medium was significantly greater than that from SHR (96.96 ± 15.3 vs. 58.11 ± 8.9 μmoles calcium/g protein/10 min; N = 10). A study of the time course of this passive

Table 5. Effect of sodium azide on calcium uptake in the presence of ATP by subcellular fractions from NWR and SHR mesenteric arteries

Fraction	5 mM ATP	5 mM ATP + 0.5 mM NaN
N_1 (PM)	15.47 ± 0.39	15.87 ± 0.77
S_1 (PM)	*20.25 ± 0.79	*20.70 ± 0.80
N_2 (ER)	7.86 ± 0.16	7.45 ± 0.33
S_2 (ER)	9.77 ± 0.65	8.47 ± 0.37
N_3	8.63 ± 1.16	7.50 ± 1.08
S_3	9.20 ± 0.20	9.16 ± 0.23

Values are expressed as μmoles calcium taken up g^{-1} protein in 10 min.

* Denotes values significantly different from those obtained for the corresponding fractions of NWR arteries.

N = 4

Table 6. Effects of ionophores and SKF-525A on calcium uptake by subcellular fractions from NWR and SHR mesenteric arteries

Fraction	Control	95% Ethanol 1%	X-537A 20 μM	A23187 10 μM	SKF-525A 100 μM	No ATP
N_1 (PM)	17.4 ± 1.2	17.0 ± 0.4	†12.6 ± 1.2	†10.4 ± 1.0	†9.3 ± 1.3	†4.4 ± 0.2
			(-4.5 ± 1.1)	(-6.7 ± 1.1)	(-7.7 ± 0.9)	(-12.7 ± 0.3)
S_1 (PM)	23.3 ± 2.1	22.0 ± 0.8	†9.7 ± 1.1	†9.9 ± 0.6	†12.6 ± 0.9	†4.9 ± 0.1
			*(-12.3 ± 1.6)	*(-12.1 ± 0.5)	(-9.5 ± 0.4)	*(-17.2 ± 0.8)
N_2 (ER)	10.5 ± 0.5	10.6 ± 0.4	†7.8 ± 0.8	9.7 ± 0.5	†7.5 ± 0.7	†2.6 ± 0.1
			(-2.7 ± 0.8)	(-0.9 ± 0.9)	(-3.0 ± 0.4)	(-7.9 ± 0.4)
S_2 (ER)	12.5 ± 1.0	12.0 ± 0.5	†6.9 ± 0.9	†8.2 ± 0.4	†7.6 ± 1.3	†2.3 ± 0.1
			*(-5.2 ± 0.6)	*(-3.9 ± 0.2)	(-4.5 ± 1.1)	(-9.7 ± 0.5)

Values are expressed as μmoles calcium taken up g^{-1} protein in 10 min. N = 4. Figures in parentheses below the related values are the differences of those values from 1% of 95% ethanol in the same fractions.

* Denotes values significantly different from those obtained under the same conditions for the corresponding fraction of NWR arteries.

+ Denotes values significantly different from those obtained from 1% of 95% ethanol in the fractions.

calcium uptake shows that both PM fractions reached maximum at about 3 to 10 min (fig. 3). The amount of calcium taken up by N_1 and S_1 was significantly different at 3, 10 and 20 min. Calcium uptake by both PM fractions was near half maximal at 1 min.

c) Comparisons of effects of diazoxide, propranolol and SKF-525A on active calcium uptake.

Diazoxide and propranolol (at 1 mM) had no significant effect on active calcium uptake by the subcellular fractions from NWR and SHR arteries (Table 7). SKF-525A decreased calcium uptake by the subcellular fractions from SHR arteries more than those from NWR arteries, but the residual calcium uptake by these subcellular fractions was not significantly different (Table 6).

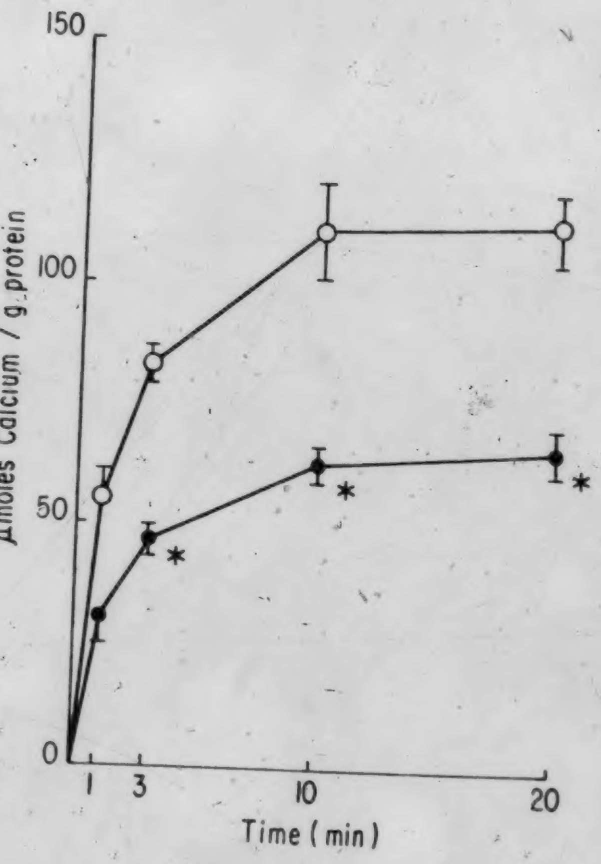

Fig. 3. Time course of passive calcium uptake by plasma membrane fractions from mesenteric arteries of NWR (open circles) and SHR (closed circles) at 1 mM calcium concentration. Asterisk indicates a significant difference from normotensive value. N = 4.

TABLE 7. Effects of diazoxide and propranolol on calcium uptake by subcellular fractions from NWR and SHR mesenteric arteries

Fraction	Control	Diazoxide (1 mM)	Propranolol (1 mM)
N_1(PM)	18.38±1.47	18.52±0.13	14.78±2.19
S_1(PM)	23.61±0.55	24.06±0.95	23.26±1.03
N_2(ER)	7.80±0.47	7.81±0.29	6.83±0.34
S_2(ER)	8.68±1.20	8.47±0.50	7.28±0.60

Values are expressed as μ moles calcium taken up g^{-1} protein in 10 min. N = 3.

D. Discussion

a) Enzymes in SHR mesenteric arteries.

On the basis of studies of enzyme activities in homogenates, and of histochemical studies, several workers have reported that AMPase, ADPase, ATPase (Ichijima, 1969; Oka and Angrist, 1965), and alkaline phosphatase (Gardner and Laing, 1965; Ooshima, 1973) are increased in arteries of hypertensive animals compared to normotensive ones. I have confirmed these results by my study of enzymatic activities in subcellular fractions. In addition, I found that phosphodiesterase I, leucine aminopeptidase, and ouabain-sensitive K^+-activated phosphatase activities were also increased in the PM fraction from SHR arteries. Since all of these enzymes are believed to be located mainly in the plasma membrane of most cell types (DePierre and Karnovsky, 1973; Touster et al., 1970), it is clear that the plasma membrane of vascular smooth muscle of SHR has undergone some biochemical changes associated with the development of hypertension. Hence the vascular wall of mesenteric arteries is biochemically abnormal.

Many of these enzymatic changes may relate to increased cell proliferation, and are the expected results for blood vessels undergoing wall thickening in response to hypertension (Weiss, 1974). For example, increased AMPase was reported in proliferating muscle cells of arterial walls (Oka and Angrist, 1967), and both AMPase and phosphodiesterase I are involved in catabolism of RNA (Yannarell and Aronson 1973).

K^+-activated phosphatase is generally believed to be a part of $(Na^+ + K^+)$-ATPase (Glynn, 1968), and therefore its biological function is related to Na^+ and K^+ transport. Jones (1974) reported that SHR arteries exhibited a decreased ability to accumulate K^+ and extrude Na^+, and increased ionic permeabilities to Na^+, K^+ and Cl^-. On the contrary, I found that the specific activity of this enzyme was increased in SHR arterial membrane fractions. My results are difficult to reconcile with the hypothesis that altered active Na^+ and K^+ transport in hypertensive arteries is secondary to reduced $(Na^+ + K^+)$-ATPase. Perhaps this ATPase activity is increased to compensate for either increased ion permeabilities or partial uncoupling of ATP utilization and ion transport.

For most of the enzymes showing increased activity in membranes of hypertensive arteries, no function can so far be assigned; thus the significance or consequence of increased activities is obscure.

b) Calcium uptake in SHR arteries.

One of the important findings in this study is that active calcium uptake by the PM fraction from SHR arteries was increased relative to that of NWR. Neither oxalate nor azide affected the calcium uptake by the PM fraction from either NWR or SHR arteries, but oxalate did increase calcium uptake by ER fractions from both animals. Since the increase of active calcium uptake by the PM fraction from SHR arteries still existed in the presence of oxalate and azide, it is most likely a property of plasma membrane.

Calcium uptake in the presence of ATP and ionophores was not significantly different in the PM fractions from NWR and SHR. Thus, ionophores may have eliminated the extra calcium transport by the plasma membrane that is associated with hypertension. Ionophores are usually postulated to act by providing a leak pathway for calcium accumulated within membrane vesicles against a concentration gradient (Scarpa et al., 1972). If this interpretation is correct, plasma membrane of hypertensive arteries may be capable of transporting more calcium out of the cell than the plasma membrane of normotensive arteries. Alternately, the plasma membrane isolated from the hypertensive animals may be less leaky to calcium. In either case, the absence of an oxalate effect reflects lack of oxalate permeation into the vesicles, not failure of accumulation of calcium within the vesicle. Recently Entman et al.(1973) have suggested that ionophores act in cardiac sarcoplasmic reticulum by interfering with an ATP-dependent calcium binding site. Ionophores and SKF-525A may inhibit calcium binding in arterial plasma membrane and differentially in membranes from arteries of hypertensive rats; but no direct evidence is available on this point.

Passively bound calcium exerts an important control over plasma membrane excitability and permeability (Triggle, 1972). I examined calcium uptake (presumably binding) by plasma membrane from a concentration of 1 mM calcium in the absence of ATP. Calcium uptake by PM fraction of SHR arteries was decreased compared to NWR. Reduced binding of calcium might be associated with a decreased membrane stability, and an increased permeability to calcium and other ions (Triggle, 1972). As mentioned above, evidence that changes in Na^+, K^+ and Cl^- permeability occur in SHR aorta has been reported (Jones, 1974). If there is also an increased permeability to calcium, the observed greater ATP-dependent calcium uptake by the SHR plasma membrane might be an expected compensatory response. Alternately, if passive binding of calcium occurs inside the vesicles, plasma membrane vesicles from arteries of SHR may have a lower permeability to calcium than similar vesicles from arteries of NWR; then their observed greater uptake of calcium in the presence of ATP as well as the greater loss of this calcium uptake in the presence of ionophores may reflect a less "leaky" vesicular fraction.

c) Other aspects.

Several investigators have emphasized that one should use multiple strains of normotensive Wistar rats as controls for SHR (Clineschmidt et al., 1970; Shibata et al., 1973). The results shown were obtained using normotensive Wistar rats from Woodlyn Farm (Ontario, Canada) as the controls. However, similar results were also obtained (see Chapter V) using a different strain of normotensive Wistar rats from Simonsen Laboratories (Gilroy, California, U.S.A.). Levy (1973) reported that Simonsen Wistar rats and Kyoto normotensive rats from which the SHR was developed had identical vascular response to pressor agents. ATP-dependent calcium uptake by the PM fraction from SHR was significantly higher than that from NWR mesenteric arteries. In contrast, the ATP-dependent calcium uptake by the plasma membrane fraction from SHR aortae was decreased as compare to that of NWR (see Chapter VI). This qualitative difference might reflect a difference in the mechanisms controlling contraction and relaxation of large and small vessels. It is well known that the contractile response of strips of "resistance arterioles" is rapid and is followed by a secondary partial relaxation (Bohr and Goulet, 1961), while the response of aortic strips is slow but sustained. The different properties of contractile response, and of calcium accumulation by the subcellular fractions observed in large and small vessels of SHR relative to NWR might suggest that the mechanisms responsible for the reactivity change in SHR aorta might be different from that of mesenteric artery. The lack of similar changes in calcium uptake by plasma membrane fractions from aortae and mesenteric arteries may also be related to the fact that contractile hyper-reactivity in the aorta (Field et al., 1972; Janis and Triggle, 1973) develops much later than in mesenteric artery (Haeusler and Finch, 1972).

In this study, alterations in factors which may control the reactivity of vascular smooth muscle in SHR have been demonstrated. Calcium uptake and ATPase were altered and these changes might be directly involved in reactivity changes in hypertensive arteries. In addition, increased activity of enzymes which are believed to be involved in cell proliferation was associated with hypertension. This indicates that changes in both calcium regulation and vessel wall thickness may be important for the development of vascular hyper-reactivity.

E. Summary

Calcium uptake and enzymatic activities were studied on plasma membrane enriched and other subcellular fractions from mesenteric arteries of normotensive Wistar (NWR) and spontaneously hypertensive rats (SHR). The specific activities of plasma membrane marker enzymes (5'-nucleotidase, phosphodiesterase I, leucine aminopeptidase, and ouabain-sensitive K^+-activated phosphatase) were significantly greater in the plasma membrane enriched fraction from arteries of hypertensive than in those from normotensive rats. Alkaline phosphatase and Mg^{++}-ATPase exhibited increased activities in all subcellular fractions of arteries from SHR as compared to those from NWR. The maximum calcium uptake in the presence of 5 mM ATP and 17 μM free calcium by the plasma membrane fraction from SHR arteries was significantly greater than that from NWR arteries. In contrast, the maximum ATP-independent calcium uptake in the presence of 1 mM free calcium by the plasma membrane fraction from SHR arteries was significantly lower than that from NWR arteries. The ionophore-induced decrease in calcium uptake by these fractions was 1.5 to 2.5 times greater for SHR than for NWR arteries. The presence of changes in activities of enzymes believed to be involved in cell proliferation as well as changes in calcium uptake suggest that increased vascular reactivity associated with hypertension is due to changes in both calcium metabolism and vessel wall thickness.

CHAPTER IV

ALTERATIONS IN CALCIUM TRANSPORT AND BINDING BY THE PLASMA MEMBRANE OF MESENTERIC ARTERIES FROM SPONTANEOUSLY HYPERTENSIVE RATS

CHAPTER IV: ALTERATIONS IN CALCIUM TRANSPORT AND BINDING BY THE PLASMA MEMBRANE OF MESENTERIC ARTERIES FROM SPONTANEOUSLY HYPERTENSIVE RATS

A. Introduction

Spontaneously hypertensive rats (SHR) are probably the best animal model for human essential hypertension (Okamoto, 1972). Perfused vasculature (Haesuler and Finch, 1972; Lais and Brody, 1975) as well as isolated strips of vessels from these rats (Field et al., 1972; Holloway and Bohr, 1973; Shibata et al., 1973) exhibit an increased sensitivity to some contractile agents. Furthermore, it has been demonstrated that certain of these alterations in vascular responsiveness of SHR can occur in the absence of increased wall stress (Hansen and Bohr, 1975). These authors have postulated that structural changes are secondary to the increase in transmural pressure whereas certain functional changes in smooth muscle sensitivity are not. The above results support the hypothesis that supersensitivity of resistance vessels to constrictor agents contribute to the initiation and maintenance of hypertension.

A derangement of calcium regulation is a plausible cause of this supersensitivity in SHR (Field et al., 1972; Janis and Triggle, 1973). However, the meaningful study of calcium movements in intact smooth muscle is very difficult or impossible, even when the "La^{+++} technique" is used (Daniel and Janis, 1975). Therefore, it was desirable to use subcellular fractions for the study of calcium uptake by organelles from blood vessels to avoid the problems associated with the use of intact smooth muscle.

It is not possible at the present time to isolate a large mass of true resistance vessels for the preparation of subcellular fractions; mesenteric arteries were used because of the relatively small size of the ileal and jejunal arteries that make up much of this preparation (see Chapter II). A plasma membrane enriched fraction isolated from mesenteric arteries of SHR exhibited decreased passive uptake of calcium when compared to that isolated from normotensive rats (NWR) (see Chapter III). This was of particular interest because Hansen and Bohr (1975) have suggested that a decreased number of calcium binding sites in plasma membrane might be the cause of increased vascular excitability associated with hypertension.

Other studies from Bohr's laboratory have shown that the reactivity of arteries to Ba^{++}, Sr^{++} and Mn^{++} was qualitatively and quantitatively different when SHR and NWR were compared (Bohr, 1974; Holloway and Bohr, 1973). These differences in reactivity to nonphysiological cations was also seen in pre-hypertensive SHR (Shibata et al., 1973), and therefore may not be secondary to hypertension. The aims of the present study were to determine whether the effects of these cations on membranal calcium uptake could be correlated to their differential contractile effects, and to use these cations as probes of the membranes from SHR to determine if other alterations of calcium binding sites are present.

B. Methods and Materials

Animals and Fraction Preparation

The male SHR of the strain developed by Okamoto and Aoke (1963) from Japanese Wistar rats were studied. Male Woodlyn Farm (Ontario, Canada) normotensive rats were used as controls. A comparative study revealed (see Chapter V) that these and male California normotensive rats (Simonsen Laboratories, Gilroy, California) were similar in all respects studied and showed similar differences from hypertensive animals. The animals were 5-6 months old and had a blood pressure of 115-130 and 170-210 mm Hg for normotensive and SHR respectively. Systolic blood pressure was recorded from the tail of unanesthesized rat with a pneumatic pulse transducer and electrosphygmograph as previously described (see Chapter III).

The animals were killed by a blow on the head and mesenteric arteries quickly removed and freed from fat, connective tissue, and veins. Plasma membrane enriched fraction from NWR and SHR was isolated by the use of a discontinuous sucrose gradient as previously described (see Chapter II).

Calcium uptake in the presence of ATP was carried out in 1.0 ml reaction mixture containing 100 mM KCl, 5 mM $MgCl_2$, 5 mM ATP (disodium or magnesium salt), 0.1 mM $CaCl_2$ labelled with 0.4 uCi $^{45}Ca\ Cl_2$, 20-40 ug membrane protein, 40 mM imidazole buffer at pH 7.0, which yielded 17 uM free calcium. Calcium uptake in the absence of ATP was studied in a 1.0 ml reaction mixture that contained: 100 mM NaCl, 1 mM

$MgCl_2$, 1 mM $CaCl_2$ labelled with 0.8 uCi $^{45}CaCl_2$, 30-60 ug membrane protein, 40 mM imidazole buffer at pH 7.0. The active and passive calcium uptake by the membrane fractions was studied by the use of the millipore filtration technique as previously described (see Chapter II).

Values were compared by the Student's t-test. Differences with p values < 0.05 were considered significant. Means and standard errors are given in the tables.

Materials

$BaCl_2$, $SrCl_2$, $MnCl_2$, $CaCl_2$ and other compounds were dissolved in 40 mM imidazole buffer at pH 7.0. Solutions were prepared from distilled deionized water and analytical grade reagents. Organic compounds were the highest purity available from Sigma; $^{45}CaCl_2$ was obtained from Amersham-Searle.

C. Results

Comparison of effect of Ba^{++}, Sr^{++}, Ca^{++} and Mn^{++} on ATP dependent $^{45}Ca^{++}$-uptake by the PM fraction from NWR and SHR arteries.

ATP dependent $^{45}Ca^{++}$-uptake by the PM fraction from SHR arteries was significantly greater than that of NWR (Table 1). At a concentration of 1 mM, Ba^{++}, Sr^{++} or unlabelled Ca^{++}, but not Mn^{++} significantly inhibited ATP-dependent $^{45}Ca^{++}$-uptake by these fractions. The ability of these cations to compete with $^{45}Ca^{++}$ for uptake by these fractions was in the order: cold $Ca^{++} > Sr^{++} > Ba^{++}$. At 1 mM, Ba^{++} could decrease $^{45}Ca^{++}$-uptake more in the PM fraction from NWR than that of SHR. On the other hand, Sr^{++} at 1 and 10 mM, and Ca^{++} at 1 mM, could decrease $^{45}Ca^{++}$-uptake more in the PM fraction from SHR than that of NWR. The decrease in $^{45}Ca^{++}$-uptake in SHR PM fractions caused by Sr^{++},(10 mM) and Ca^{++}(1 mM) was significantly different from that of NWR membranes, and the residual amount of $^{45}Ca^{++}$ taken up were not different between the fractions from normotensive and hypertensive rats. Mn^{++} (1 mM) did not affect the active calcium uptake by the PM fractions from either SHR or NWR arteries.

Comparison of effect of Ba^{++}, Sr^{++}, Ca^{++} and Mn^{++} on ATP independent $^{45}Ca^{++}$-uptake by the PM fraction from NWR and SHR arteries.

ATP independent $^{45}Ca^{++}$-uptake by the PM fraction from SHR arteries was significantly lower than that of NWR (Table 2). At the doses used Ba^{++}, Sr^{++}, Mn^{++} and cold Ca^{++} could significantly decrease $^{45}Ca^{++}$ taken up from the PM fractions from NWR and SHR arteries. Despite

TABLE 1: Effects of divalent cations on $^{45}Ca^{++}$-uptake in the presence of ATP by plasma membrane fractions from NWR and SHR arteries

Rat	Control	Mn^{++} (1mM)	Ba^{++} (1mM)	Sr^{++} (1mM)	Ca^{++} (1mM)	Sr^{++} (10mM)
NWR	153.2±13.3	151.0±8.0	$^{+}$119.9±4.8	$^{+}$74.5±4.1	$^{+}$40.6±2.9	$^{+}$21.9±1.4
		(-2.2±6.8)	(-33.4±4.8)	(-78.8±12.1)	(-112.6±15.6)	(-131.3±13.1)
SHR	*203.5±7.7	*219.6±12.9	$^{*+}$190.2±8.2	$^{*+}$116.3±4.2	$^{+}$40.5±1.9	$^{+}$16.1±2.6
		(+16.0±6.3)	*(-13.4±2.6)	(-87.3±5.4)	*(-163.0±7.4)	*(-187.4±5.9)

Values are expressed as 10^{6} dpm g^{-1} protein in 10 min. N = 5.

Figures in parentheses below the related values are the differences of those values from control values of the same fraction.

* Denotes values significantly different from those obtained under the same conditions for the corresponding fraction of NWR mesenteric arteries.

$^{+}$ Denotes values significantly different from those obtained in controls of the same fraction.

TABLE 2: Effect of divalent cations on $^{45}Ca^{++}$-uptake in the absence of ATP by plasma membrane fractions from NWR and SHR arteries

Rat	Control	Mn^{++} (1mM)	Ba^{++} (1mM)	Sr^{++} (10mM)	Ca^{++} (10mM)
NWR	132.2±4.7	$^{+}$53.8±4.2	$^{+}$106.7±3.7	$^{+}$31.0±2.9	$^{+}$24.9±2.4
		(-78.4±7.8)	(-25.6±3.3)	(-101.2±4.3)	(-107.4±4.8)
SHR	*68.5±4.1	$^{+}$47.4±3.7	$^{*+}$27.1±3.3	$^{*+}$21.6±2.3	$^{*+}$15.3±2.9
		*(-21.2±1.2)	*(-41.4±5.7)	*(-46.9±3.1)	*(-53.2±1.7)

Values are expressed as 10^6 dpm g^{-1} protein in 10 min. N = 5.

Figures in parentheses below the related values are the differences of those values from control values of the same fraction.

* Denotes values significantly different from those obtained under the same conditions for the corresponding fraction of NWR mesenteric arteries.

$^{+}$ Denotes values significantly different from those obtained in controls of the same fraction.

this, Ba^{++} (1 mM) inhibited $^{45}Ca^{++}$-uptake by the PM fraction significantly more from SHR arteries than that from NWR. On the other hand, Mn^{++} (1 mM) inhibited $^{45}Ca^{++}$-uptake more by the PM fraction from NWR arteries than that from SHR. In large excess (10 mM) Sr^{++} and Ca^{++} reduced all $^{45}Ca^{++}$-uptake to a very low level which probably represented non-specific binding; naturally there was a greater decrease in $^{45}Ca^{++}$-uptake in fractions from NWR than that from SHR arteries. The residual amounts of calcium bound to the PM fraction from NWR were still significantly greater than those to the PM fraction from SHR in the presence of Ba^{++}, Sr^{++}, and cold Ca^{++}, but not Mn^{++}, but in all cases the difference was markedly reduced.

D. Discussion

Alterations in calcium uptake and enzymatic activities were present in subcellular fractions from mesenteric arteries of SHR (see Chapter III). The results in this study further show that there are differential effects of various cations on calcium uptake by the PM fractions from SHR and NWR arteries and support Bohr's suggestions (1974) on the reactivity changes accompanying hypertension.

The differences between the plasma membrane fractions from NWR and SHR arteries with respect to ATP-dependent calcium uptake were noted when other divalent cations were added to compete with $^{45}Ca^{++}$ during uptake. I examined competition of these cations with calcium because of the findings of Bohr (1974) and Shibata et al., (1973) on interaction of these cations with calcium in excitation-contraction coupling in intact artery strips. The concentrations of cations used were those found by Bohr (1974) to differentially affect contractions of arteries from normotensive and hypertensive rats (1 mM Ba^{++} and Mn^{++}, 10 mM Sr^{++}). In my study, the ability of Sr^{++} and Ba^{++} (1 mM) to compete for ATP-dependent sites of uptake with $^{45}Ca^{++}$ was less than that of unlabelled Ca^{++}, but Sr^{++} was more effective than Ba^{++} which was nearly ineffective. Mn^{++} was completely ineffective. Similar results were reported for Mn^{++} and Ba^{++} in microsomes from guinea pig intestinal smooth muscle (Hurwitz et al., 1975). The uptake of $^{45}Ca^{++}$ prevented by competition was greater in plasma membranes from arteries of hypertensive animals than in those from arteries of normotensive animals when either Sr^{++}(1 mM or 10 mM) or unlabelled Ca^{++} (1 mM) was added. In contrast, Ba^{++} (1 mM) was more effective in plasma membranes from arteries of normotensive animals. Mn^{++} (1 mM) did not significantly diminish uptake of calcium by plasma membrane of either type of rat. Thus the calcium uptake processes were differently susceptible to competition by other cations in plasma membranes of normotensive and hypertensive arteries.

Both Sr^{++} and ionophores (see Chapter III) displaced more $^{45}Ca^{++}$ from the plasma membrane fraction which was derived from arteries of hypertensive animals than those of normotensive animals. These results suggest that at least two types of calcium uptake sites exist in these plasma membranes and that the proportion of each type may vary in hypertension. Furthermore, these results are consistent with some of the findings of Bohr (1974) and Shibata et al., (1973) : contractions of the carotid and aortic strips to Ba^{++} were significantly higher in amplitude in NWR as compared to SHR, but contractions induced by Sr^{++} and Mn^{++} were significantly higher in amplitude for SHR than those in NWR.

These cations may cause contraction by releasing calcium from binding sites in plasma membrane, thereby triggering contraction. The ability of Ba^{++} and Sr^{++} to compete with ATP-dependent calcium uptake by PM fractions in my results was correlated with such a release. Mn^{++} did not affect active calcium uptake in these fractions suggesting that its mechanism of action to provoke contraction may be different from Ba^{++} and Sr^{++}, but see below. ATP-dependent calcium uptake is assumed to be due to the presence of inside-out vesicles in my fractions and presumably reflects the activity of an outwardly directed calcium pump in the plasma membrane. Inhibition of calcium pumping may not be functionally equivalent to release of Ca^{++} from binding. I have not studied effects of cations on release since it would be appropriate to apply the cations to the outer surface of the plasma membrane, and this is presumably the inner surface of the vesicles.

The ability of Ba^{++}, Sr^{++} and Mn^{++} to compete with $^{45}Ca^{++}$ for ATP independent uptake also shows that differences exist between plasma membrane fractions from NWR and SHR arteries. Ba^{++} (1 mM) was also as effective as 10 mM Sr^{++} in inhibiting $^{45}Ca^{++}$ uptake from plasma membrane of SHR arteries but was much less effective in this regard in membranes from NWR arteries. Ca^{++} and Sr^{++} at concentrations of 10 mM were both effective in displacing $^{45}Ca^{++}$ more effectively from the plasma membrane of NWR and SHR arteries. Mn^{++} (1 mM) displaced $^{45}Ca^{++}$ more effectively from the plasma membrane of NWR arteries than of SHR arteries, and residual $^{45}Ca^{++}$ uptake was not the same for both types of membranes. These results show that ATP independent sites of $^{45}Ca^{++}$ uptake differ from ATP dependent ones: first, Mn^{++} (1 mM) did not affect $^{45}Ca^{++}$ uptake in the presence of ATP but it did reduced $^{45}Ca^{++}$ uptake in its absence; and second, Ba^{++} was a much more effective competitor relative to Sr^{++} at least in membranes from SHR in the absence of ATP. My findings were inconsistent with any straightforward explanation of results of Bohr, Shibata and co-workers in terms of contraction being initiated by displacement of Ca^{++} by cations from ATP independent binding sites. In fact, there is an inverse correlation between these results and those predicted on this mechanism.

Electron microscopic studies have shown that the plasma membrane fractions are composed of vesicles (see Chapter II). It is possible that more of the vesicles from SHR arteries are of the inside-out orientation than those from NWR arteries. Therefore, the effects of competing cations might be due to (a) alteration in binding properties of the exposed sites, (b) alteration in the transport to the unexposed sites, or (c) alteration in the proportion of inside-out and right side-out vesicles. This may be related to the marked effect of Ba^{++} on inhibiting ATP independent calcium uptake by vesicles of SHR arteries, and to the increased effectiveness of Mn^{++} in competing with this passively bound calcium.

The interpretation of results obtained for $^{45}Ca^{++}$-uptake in the presence of ATP is complicated by the fact that the added cations competed not only for $^{45}Ca^{++}$ uptake sites in membranes, but also with Mg^{++} and $^{45}Ca^{++}$ for complexing with ATP. However, there was 5 mM each ATP and Mg^{++} and calculations have shown that this provides an excess of Mg-ATP for activation of Ca^{++} binding. Thus the 1 mM concentrations of competing cations would probably not inhibit the utilization of ATP for $^{45}Ca^{++}$ binding. In the presence of 10 ~~mM~~ Sr^{++}, Mg^{++} was substantially displaced from the ATP complex. Also the order of affinities of cations for ATP was $Mn^{++} > Mg^{++} > Ca^{++} > Sr^{++} > Ba^{++}$ (Phillips, 1966; Izatt et al., 1971). There is some possibility that the affinity of the active site for Mg-ATP utilization may have a much higher affinity for another ATP-cation complex than for Mg-ATP; if so, cations may have inhibited $^{45}Ca^{++}$-uptake by preventing ATP utilization for the process. Complexing of added cations by ATP reduced their concentrations, but this can not explain the ineffectiveness of Ba^{++} in displacing Ca^{++} since less of this cation was complexed than Sr^{++} and Ca^{++}. However, Mn^{++} concentrations were substantially reduced in the presence of ATP because of the higher affinity of this cation than of Mg^{++} for ATP. This may explain the ineffectiveness of Mn^{++} as a competitor for ^{45}Ca-uptake in the presence of ATP. The general effect of adding a cation with an affinity for ATP would be to increase the level of free $^{45}Ca^{++}$, but the increases would be small and similar except when 10 mM Sr^{++} was added. It is important to emphasize that none of the above considerations will account for a difference in the effect of competing cation on NWR and SHR; these comparisons were made under identical conditions.

The results of this study show that the susceptibility of the calcium uptake processes to competition by other cations is different in plasma membranes of normotensive and hypertensive arteries. This further supports the view that supersensitivity of the reactivity of vascular smooth muscle of SHR to contractile agents may be due to a derangement in calcium regulation. Furthermore, it is possible that a change of calcium regulation is responsible for the thickening of vascular walls of hypertensive patients (Folkow, 1971) because calcium also plays a number of key roles in cellular metabolism, including the regulation of important phosphoproteins and of stimulus-division coupling (Berridge, 1975).

E. Summary

The effect of Sr^{++}, Ba^{++} and Mn^{++} on calcium uptake by a plasma membrane enriched fraction from mesenteric arteries of spontaneously hypertensive rats (SHR) was studied to determine what alterations in divalent cation interactions are associated with hypertension. ATP-dependent $^{45}Ca^{++}$ uptake by this fraction from SHR was significantly greater than that of normotensive rats (NWR). This $^{45}Ca^{++}$ uptake by these fractions from both types of rat could be reduced by Ba^{++}, Sr^{++} and non-radioactive Ca^{++}, but not by Mn^{++}; the ability of these cations to compete with $^{45}Ca^{++}$ was in the following order: $Ca^{++} > Sr^{++} > Ba^{++}$. In contrast, ATP-independent $^{45}Ca^{++}$ uptake by this subcellular fraction from SHR was significantly less than that of NWR, and this passive $^{45}Ca^{++}$ binding could be displaced by Mn^{++} in addition to the other cations. Both qualitative and quantitative differences were found between SHR and NWR in this study of the effects of cations on calcium uptake by arterial plasma membrane. These results suggest that the plasma membrane of SHR arteries has calcium binding and transport properties that are different from those of NWR. However, there is not a complete correlation between the reported effects of the cations on contraction of other arteries in hypertension and on calcium uptake by membranes of SHR and NWR.

CHAPTER V

RELATIONSHIP BETWEEN BLOOD PRESSURE OF SPONTANEOUSLY HYPERTENSIVE RATS AND ALTERATIONS IN MEMBRANE PROPERTIES OF MESENTERIC ARTERIES

CHAPTER V: RELATIONSHIP BETWEEN BLOOD PRESSURE OF SPONTANEOUSLY HYPERTENSIVE RATS AND ALTERATIONS IN MEMBRANE PROPERTIES OF MESENTERIC ARTERIES

A. Introduction

I found that the specific activities of certain enzymes of a plasma membrane enriched fraction isolated from mesenteric arteries (see Chapter II) of spontaneously hypertensive rats (SHR) were greater than those of Ontario normotensive Wistar rats (NWRo) (see Chapter III). Furthermore, maximum ATP-dependent calcium uptake under certain conditions was greater for this fraction from SHR than that from NWRo (see Chapter III). There is no ideal control for SHR and therefore more than one strain of normotensive rat should be used in studies of this type. In the present investigation I have studied enzymatic and calcium uptake parameters in a second strain, California Wistar normotensive rats (NWRc). Levy (1973) has reported that this strain exhibits a vascular response to pressor agents that is identical to that of the Kyoto normotensive rats from which the SHR was developed. Therefore, one aim of this study was to evaluate whether the differences previously reported (see Chapter III) between 5-6 month old SHR and NWRo were due to strain differences unrelated to hypertension.

Two other types of experiments were also carried out in the present investigation to further characterize the relationship between hypertension and the alterations in the above-mentioned membrane parameters. First, SHR and NWRc were compared at 3 age groups to determine if enzymatic activities or calcium uptake were abnormal before as well as after the development of hypertension. Secondly, these same parameters were studied in mesenteric arteries from rats treated with the antihypertensive agent, hydralazine, to determine if the prevention of hypertension would also prevent the development of differences between SHR and normotensive rats.

B. Methods and Materials

Animals

The male SHR (Okamoto and Aoki, 1963) used in this investigation were bred at our facilities, and age-matched male NWRc were supplied by Simonsen Laboratories, Gilroy, California, and NWRo by Woodlyn Farms, Ontario. Hydralazine treatment of SHR and NWRc was started at less than one month of age. The rats were fed, ad libitum, 80 mg/liter of drinking water during the first month, and then 100 mg/liter during the next 2-3 months.

The systolic blood pressure was monitored by using a Narco-Bio-Systems Model PE-300 sphygmomanometer for indirect measurement of tail artery pressure as previously described (see Chapter III). Hypertension was arbitrarily defined in this study as systolic blood pressure greater than 150 mm Hg.

Subcellular fractions

The animals were killed by a blow on the head and mesenteric arteries quickly removed and freed from fat, connective tissue and veins. Plasma membrane and endoplasmic reticulum fractions from NWR and SHR were isolated by the use of a discontinuous sucrose density gradient as previously described (see Chapter III).

Enzyme assays and calcium uptake studies

Protein (Lowry et al., 1951), phosphate (Fiske and SubbaRow, 1925), 5'-nucleotidase, Mg^{++}-ATPase and alkaline phosphatase were determined as described (see Chapters II and III). Calcium uptake in the presence of ATP was carried out in a reaction mixture containing 100 mM KCl, 5 mM $MgCl_2$, 5 mM ATP (disodium salt), 0.1 mM $CaCl_2$ labelled with 0.4 μCi $^{45}CaCl_2$, 15-25 μg membrane protein, 40 mM imidazole buffer at pH 7.0 in a total volume of 1 ml which yields a 17 μM free calcium concentration. Millipore filtration technique was used to determine ^{45}Ca uptake (see Chapter II).

Reagents and drugs

Hydralazine HCl was supplied by Sigma. The divalent cation ionophore, X-537 A, (supplied by Hoffman-LaRoche) was dissolved in 95% of ethanol and 10 μl of the drug was added to a total of 1 ml of reaction mixture. The other reagents and drugs were dissolved in 40 mM imidazole buffer at pH 7.0. Solutions were prepared from distilled deionized water and analytical grade reagents. Organic compounds were the highest purity available from Sigma; $^{45}CaCl_2$ was obtained from Amersham-Searle.

Statistical test

Values were compared by the Student's t-test. Differences with P value < 0.05 were considered significant. Standard errors of the means are bracketed in the figures.

C. Results

Comparison of the two strains of normotensive rats

There were no significant differences between the 5-6 month old NWRo and NWRc strains of rat in the parameters measured. This was true for both body weight and blood pressure as well as for measurements made on the subcellular fractions: 5'-nucleotidase, Mg^{++}-ATPase, alkaline phosphatase and calcium uptake (Table 1). Therefore, the quantitative and qualitative differences in the above parameters between NWRc and SHR at this age were similar to the differences between NWRo and SHR that were described previously (see Chapter III).

Comparison of hypertensive and normotensive rats at different ages

The mean body weight of SHR was the same as that of NWRc at 33 days of age, but was significantly less than that of NWRc at both 3-4 months and 5-6 months of age. In contrast, the mean blood pressure of SHR was significantly greater than that of NWRc even at an age of 33 days (Table 2). In general, the specific activities of 5'-nucleotidase and Mg^{++}-ATPase in the plasma membrane fraction of arteries from NWRc decreased significantly with age, especially between ages 3-4 months and 5-6 months (Table 3). However, the activities of these enzymes did not change in SHR over this total period; there was a small, temporary reduction in activities at 3-4 months. No significant decreases in enzyme activities of endoplasmic

TABLE 1

Comparisons of body weight, blood pressure, enzyme activities, and calcium uptake of subcellular fractions from two normotensive strains of rats at age 5-6 months[1].

Parameter	Strain	
	NWRo	NWRc
Body weight (g)	482 ± 4.0	490 ± 5.0
Blood pressure (mm Hg)	125 ± 5.5	126 ± 2.0
5'-nucleotidase		
PM[2]	58.4 ± 3.9	58.6 ± 1.4
ER	9.5 ± 1.1	10.8 ± 0.9
Mg^{++}-ATPase		
PM	361 ± 16	371 ± 23
ER	147 ± 11	138 ± 16
Alkaline phosphatase		
PM	7.7 ± 0.3	8.1 ± 0.6
ER	2.1 ± 0.3	2.5 ± 0.5
Calcium uptake		
PM	17.4 ± 1.2	17.2 ± 0.9
ER	10.5 ± 0.5	10.9 ± 0.5

[1] Specific activities are expressed as µmoles phosphate released mg^{-1} protein h^{-1} for 5'-nucleotidase, and Mg^{++}-ATPase, as µmoles p-nitrophenol released mg^{-1} protein h^{-1} for alkaline phosphatase and as µmoles calcium taken up g^{-1} protein in 10 min for calcium uptake. N = 4-5 except the first two parameters, N= 30-40.

[2] PM and ER refer to the plasma membrane and endoplasmic reticulum enriched fractions, respectively.

TABLE 2

Body weights and blood pressures of normotensive and hypertensive rats in different age groups.

	Age		
	33 days	3-4 months	5-6 months
Body weight (g)			
NWRc	86.8 ± 1.5	408 ± 6.4	490 ± 5.0
SHR	83.8 ± 2.2	*317 ± 12.4	*350 ± 3.5
Blood pressure (mm Hg)			
NWRc	116 ± 2.0	125 ± 1.3	126 ± 2.0
SHR	*133 ± 2.2	*179 ± 5.0	*196 ± 5.0

There were 20 rats in each group.

*Denotes significant difference from normotensive rat.

TABLE 3

Specific activities of 5'-nucleotidase, Mg^{++}-ATPase and alkaline phosphatase, and the capacity of calcium uptake in the plasma membrane fraction of NWRc and SHR arteries.

	Age		
	33 days	3-4 months	5-6 months
5'-Nucleotidase			
NWRc	69.78 ± 0.95	66.78 ± 2.69	†58.59 ± 1.40
SHR	73.44 ± 1.38	†67.40 ± 2.81	*70.36 ± 2.00
Mg^{++}-ATPase			
NWRc	497.8 ± 20.2	488.4 ± 16.6	†371 ± 23.3
SHR	544.9 ± 20.8	†490.3 ± 16.7	*535 ± 19.7
Alkaline phosphatase			
NWRc	6.47 ± 0.54	†7.55 ± 0.30	†8.10 ± 0.60
SHR	*7.83 ± 0.33	*†12.89 ± 0.93	*†14.30 ± 1.11
Calcium uptake			
NWRc	16.70 ± 0.42	17.27 ± 0.50	17.16 ± 0.91
SHR	17.25 ± 0.63	17.78 ± 0.88	*†22.37 ± 0.77

Values are expressed as in Table 1.

*Denotes significantly different from normotensive.

†Denotes significant differences from values at 33 days.

N = 4.

reticulum occurred with age in NWRc, but in SHR, Mg^{++}-ATPase activity was increased at 5-6 months (Table 4). Therefore, the specific activities of both 5'-nucleotidase and Mg^{++}-ATPase were significantly greater in the plasma membrane fraction, but only the latter enzyme was greater in the endoplasmic reticulum fraction of 5-6 month old SHR when compared with NWRc.

In contrast to the decrease of 5'-nucleotidase and Mg^{++}-ATPase of the plasma membrane fraction with age in NWRc but not in SHR, alkaline phosphatase and calcium uptake capacity increased with age in SHR more than in NWRc in this fraction (Table 3). However, alkaline phosphatase and calcium uptake of the endoplasmic reticulum fraction from both strains of rat increased with age (Table 4). Alkaline phosphatase activity was initially higher in both fractions from SHR compared to NWRc and the difference increased with age. Hence, the time course of the development of increased alkaline phosphatase activity in the two fractions was similar to that of increased blood pressure in SHR over the range studied. On the other hand, calcium uptake capacity, as measured under my experimental conditions, was not significantly greater for the plasma membrane fraction of SHR relative to NWRc until after hypertension has developed for several months (Table 3).

The decrease in calcium uptake in the presence of the divalent cation ionophore, X-537 A, for both fractions from 5-6 months old SHR arteries was significantly greater than that of fractions from NWR arteries. This difference was not present at one month (Table 5) nor at 3-4 months of age (Table 7). The residual calcium uptake by

TABLE 4

Specific activities of 5'-nucleotidase, Mg^{++}-ATPase and alkaline phosphatase, and the capacity of calcium uptake in the endoplasmic reticulum fraction of NWRc and SHR arteries

	Age		
	33 days	3-4 months	5-6 months
5'-Nucleotidase			
NWRc	11.75 ± 1.00	11.01 ± 0.75	10.84 ± 0.85
SHR	11.54 ± 0.88	10.92 ± 0.54	10.96 ± 1.44
Mg^{++}-ATPase			
NWRc	138.4 ± 16.7	135.5 ± 5.15	138.2 ± 6.2
SHR	144.1 ± 13.3	137.9 ± 4.06	*†185.1 ± 15.1
Alkaline phosphatase			
NWRc	1.69 ± 0.15	1.50 ± 0.30	†2.50 ± 0.50
SHR	*2.07 ± 0.21	*†3.51 ± 0.32	*†6.37 ± 0.60
Calcium uptake			
NWRc	8.66 ± 0.66	9.38 ± 0.38	†10.99 ± 0.56
SHR	8.71 ± 0.42	†10.35 ± 0.45	†11.69 ± 1.22

Values are expressed as in Table 1. N = 4.

TABLE 5

Effect of X-537 A on calcium uptake by subcellular fractions from NWRc and SHR mesenteric arteries.

Fraction	Control	95% Ethanol 1%	X-537 A 20 μM
Plasma membrane			
33 days NWRc	16.70 ± 0.42	16.45 ± 0.25	10.72 ± 0.39
SHR	17.25 ± 0.63	16.78 ± 0.62	10.06 ± 0.33
5-6 months NWRc	17.16 ± 0.91	16.87 ± 0.51	9.04 ± 0.59 (7.83 ± 0.20)
SHR	*22.37 ± 0.77	*22.75 ± 1.37	9.72 ± 0.46 (*13.03 ± 1.37)
Endoplasmic reticulum			
33 days NWRc	9.66 ± 0.66	9.86 ± 0.23	6.10 ± 0.69
SHR	9.77 ± 0.42	9.55 ± 0.38	6.02 ± 0.59
5-6 months NWRc	10.99 ± 0.56	11.40 ± 0.92	8.00 ± 0.58 (3.35 ± 0.60)
SHR	11.69 ± 1.22	11.50 ± 1.08	7.02 ± 0.62 (*4.50 ± 0.56)

Values are expressed as umoles calcium taken up g^{-1} protein in 10 min. N = 4. Figures in parentheses below the related values are the differences of those values from 1% of 95% ethanol in the same fractions.

*Denotes values significantly different from those obtained under the same conditions for the corresponding fraction of NWR arteries.

subcellular fractions of arteries in the presence of ATP and X-537 A was not different at different ages from NWRc or SHR (Tables 5 and 7).

Effect of hydralazine treatment

Treatment with hydralazine for approximately 3 months did not affect body weight of either strain of rat, but it did decrease blood pressures of both SHR and NWRc; this decrease was more marked for the hypertensive rats (Figure 1). The blood pressure of hydralazine-treated SHR was slightly but significantly higher than that of age-matched untreated NWRc (Figure 1).

The specific activity of alkaline phosphatase (Figure 2) in the subcellular fractions of 3-4 months old SHR was significantly greater than that of NWRc. Treatment with hydralazine partially prevented the increase in the specific activity of alkaline phosphatase which occurred between 33 days and 3-4 months in both fractions from SHR (Figure 2, and Tables 3 and 4). The specific activity of alkaline phosphatase in the plasma membrane fraction from NWRc arteries also increased slightly with age (Table 3). After hydralazine treatment, this enzyme activity in the plasma membrane fraction from NWRc arteries at 3-4 months old decreased to a level not significantly different from that at 33 days (Figure 2, and Table 3). The specific activity of alkaline phosphatase in the plasma membrane and endoplasmic reticulum fractions of hydralazine-treated SHR did not differ from that of untreated NWRc (Figure 2). Similarly, the specific activity of Mg^{++}-ATPase and 5'-nucleotidase (Table 6) in the subcellular fractions from 3-4 months old rats did not

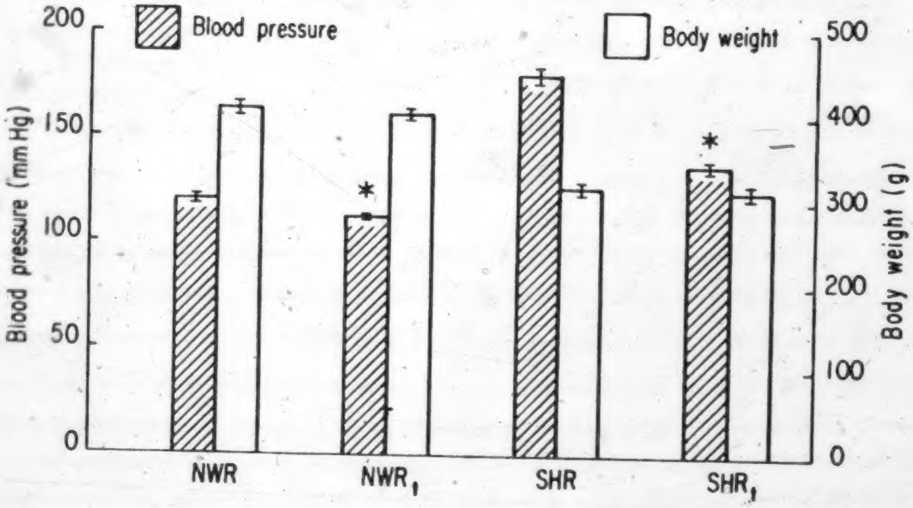

FIGURE 1 Body weights and blood pressures of hydralazine-treated (NWRt and SHRt) and untreated 3-4 month old rats.^{x}

^{x}All of the SHR and SHRt values are significantly different from those of both NWR and NWRt.

*Denotes significance from untreated rat of the same strain.

N = 4.

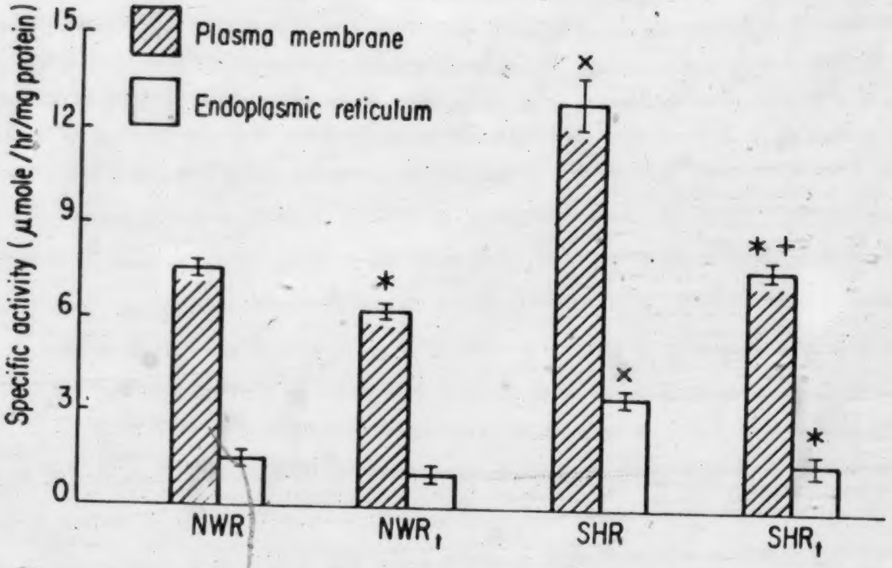

FIGURE 2 Specific activity of alkaline phosphatase in subcellular fractions of hydralazine treated (NWRt and SHRt) and untreated rats.

*Denotes significance from untreated rat of the same strain.

×Denotes significance from both NWR and NWRt.

†Denotes significance from NWRt.

N = 4.

TABLE 6

Specific activities of 5'-nucleotidase and Mg^{++}-ATPase in subcellular fractions from hydralazine treated (NWRt and SHRt) and untreated 3-4 month old rats.

Enzyme and Type of Rat	Fraction	
	Plasma membrane	Endoplasmic reticulum
5'-Nucleotidase		
NWR	66.78 ± 2.69	11.01 ± 0.75
NWRt	67.00 ± 3.36	11.45 ± 0.80
SHR	67.40 ± 2.71	10.92 ± 0.54
SHRt	66.98 ± 4.42	10.43 ± 0.83
Mg^{++}-ATPase		
NWR	488.4 ± 16.6	135.45 ± 5.15
NWRt	483.9 ± 12.6	138.30 ± 5.97
SHR	492.3 ± 16.7	137.9 ± 4.06
SHRt	488.0 ± 15.7	134.0 ± 7.13

Values are expressed as in Table 1, N = 4.

differ significantly among the four groups (Table 6); this was expected because none of these activities had changed in 3-4 months old rats with hypertension compared to the values at 33 days (Tables 3 and 4). No significant increase in calcium uptake was found in the plasma membrane or endoplasmic reticulum fractions of arteries from untreated 3-4 months old SHR as compared to that of untreated NWRc (Tables 2 and 3). Hydralazine treatment of NWRc and SHR also did not result in a significant change in calcium uptake by the subcellular fractions in either group (Table 7). ATP-dependent calcium uptake by the subcellular fractions from both NWRc or SHR arteries was decreased similarly by the presence of X-537 A (Table 7). Again, there was no significant difference between values for fractions from untreated NWRc and SHR as compared to similar fractions from hydralazine-treated animals (Table 7).

TABLE 7

Effect of X-537 A on calcium uptake by subcellular fractions from hydralazine-treated (NWRt and SHRt) and untreated 3-4 month old rats.

	Control	X-537 A (20 μM)	Inhibition of calcium uptake by X-537 A
Plasma membrane			
NWR	17.55 ± 1.00	10.76 ± 1.40	6.79 ± 1.20
NWRt	17.23 ± 0.92	11.00 ± 1.10	6.23 ± 1.10
SHR	18.57 ± 0.86	10.86 ± 0.84	7.69 ± 0.85
SHRt	18.03 ± 0.67	11.34 ± 0.72	6.69 ± 0.70
Endoplasmic reticulum			
NWR	10.76 ± 0.76	6.93 ± 1.20	3.83 ± 0.90
NWRt	10.43 ± 0.83	6.25 ± 1.05	4.19 ± 0.95
SHR	11.69 ± 0.69	7.40 ± 0.95	4.29 ± 0.75
SHRt	10.47 ± 0.80	6.80 ± 0.75	3.69 ± 0.71

Values are expressed as in Table 1. N = 4.

D. Discussion

The significant increases in the specific activities of enzymes and in the capacity of active calcium uptake of subcellular fractions from 5-6 months old SHR arteries as compared to those from NWRc confirm the results in which a different strain of control animal was used (see Chapter III). Both calcium uptake and enzyme activities were the same in these two strains of normotensive rats (Table 1). This suggests that the differences observed between SHR and the normotensive rats may be related to hypertension rather than to differences in the animal strains. Furthermore, there were only slight changes in these parameters, with the exception of alkaline phosphatase, in 33 days and 3-4 months old SHR when compared to NWRc of the same age. The 33 days SHR were pre-hypertensive by my definition (blood pressure $<$ 150 mm Hg), although their blood pressures were significantly elevated compared to NWRc, whereas 3-4 months old SHR were clearly hypertensive. Therefore, the appearance of these changes, except for that in alkaline phosphatase, followed the appearance of hypertension and may be a consequence of it. Since 5'-nucleotidase and Mg^{++}-ATPase were also increased in rats made hypertensive by salt treatment (Oka and Angrist, 1965; 1967), these changes may result from hypertension, regardless of its etiology. The time course of elevation of alkaline phosphatase activity with age differed from that of other enzymes and calcium uptake. The activities of other enzymes failed to decrease with age in SHR as they did in NWRc while alkaline phosphatase activities were already increased in SHR compared to NWRc at 33 days, and continued to increase with age. The uptake of calcium also increased with age in SHR but did not change in NWRc, however, this increase became significant only at 5-6 months.

The studies on hydralazine-treated NWRc and SHR were designed to resolve further the relationship between hypertension and these alterations. The mean systolic blood pressures of all untreated rats of the hypertensive strain at 3-4 months of age was over 150 mm Hg. However, at that age the specific activities of 5'-nucleotidase, Mg^{++}-ATPase, and the amount of calcium uptake (and ionophore decreased calcium uptake) in the subcellular fractions from SHR arteries were not significantly greater than those of NWRc. Only the specific activity of alkaline phosphatase in the plasma membrane and endoplasmic reticulum fractions of SHR arteries was significantly greater than that of NWR. Treatment with hydralazine decreased the blood pressures of SHR and NWRc, and the former was decreased much more than the latter. Similar results have been reported by another group (Sen et al., 1974). Such treatment did not significantly change the specific activities of 5'-nucleotidase, Mg^{++}-ATPase and the capacity of calcium uptake by the subcellular fractions of either from SHR or NWR arteries as compared to untreated rats. There was also no change relative to 33 day old animals. This suggests that the treatment had no permanent non-specific effect on the enzymatic activities or calcium uptake parameters studied. However, the treatment did prevent the increase in the specific activity of alkaline phosphatase in the subcellular fractions of hypertensive rat arteries. This was correlated with the prevention of the increase in blood pressure in both groups.

From this study it is clear that an increase in the specific activity of alkaline phosphatase of membranes from small blood vessels is closely associated with the increase in blood pressure, and may be related to the originating cause of hypertension. However, all the other changes I have observed in membranes from hypertensive blood vessels do appear to be secondary to increased blood pressure. The changes I have observed with respect to alkaline phosphatase activity are not unique; other enzyme activities or other biochemical parameters may show a similar relationship to hypertension. For example, abnormalities in patterns of acid phosphatase isozymes are reported in SHR liver after seven days of age but not in the fetus liver (Park, 1975).

Histochemical studies on rat arteries have demonstrated that the AMPase (5'-nucleotidase) activity is increased in the earliest stage of renal and salt hypertension (Oka and Angrist, 1965; 1967). On the other hand, Ichijima (1969) found the activity of this enzyme unchanged in one month old SHR, but increased in 4-6 months or older SHR. The alterations observed on hypertensive rats were compared to the results of age-matched normotensive ones. The reason for such a difference is not known, but this difference may be related to a difference in pathogenesis between renal or salt hypertension and SHR. My results confirm those of Ichijima on SHR.

Ooshima (1973) has reported a positive correlation between blood pressure and alkaline phosphatase activity in homogenates of the vascular system of SHR as compared to similar ages of normotensive rats. The blood pressure increase which occurs with age was diminished in hydralazine-treated SHR, and the parallel increase in alkaline phosphatase activity was similarly prevented. Therefore, my results further extend Ooshima's observation. In addition, a significant increase in the specific activity of alkaline phosphatase was found in the youngest SHR which I studied.

Little is known about the physiological function of alkaline phosphatase (Fernley, 1971). However, it would be of considerable interest to know if the phosphatase that I studied in SHR membranes has protein phosphatase activity. There is increasing evidence that phosphoproteins may play a key role in regulating Ca^{++} transport and ATPase activity as well as membrane permeability (Rubin and Rosen, 1975; Tada et al, 1975), and it is known that some alkaline phosphatases have protein phosphatase activity (Lee and Nickol, 1974). Furthermore, Sirica and coworkers (1975) have considered the possibility that decreased Mg^{++}-ATPase and 5'-nucleotidase, and increased alkaline phosphatase activities of regenerating liver plasma membrane may be associated with specific membrane transport abnormalities.

Early alterations in vascular permeability have been reported (Byrom, 1969; Giese, 1966). Of particular interest is a report by Shibata and coworkers (1973) demonstrating that increased responsiveness to strontium, lanthanum and manganese is present in the vascular smooth muscle of SHR before the onset of hypertension. In addition, Hansen and Bohr (1975) have shown that the increased responsiveness of femoral arteries from hypertensive SHR to strontium and lanthanum is not caused by hypertension via increased wall stress nor is it caused by increased wall thickness. They also found that high concentrations of calcium did not depress the response of the femoral artery from SHR, unlike that from normotensive rats. A somewhat analogous response has been reported for aortic smooth muscle from SHR (Field et al, 1972).

Lais and Brody (1975) found that perfused hindquarters of SHR were supersensitive to norepinephrine but not to barium nor angiotensin, and concluded that SHR hypersensitivity is specific. In contrast, the studies cited above as well as those of Haeusler and Finch (1972) on perfused mesenteric artery from SHR indicate that there is a somewhat non-specific supersensitivity in at least some vasculature of SHR. It is this type of reactivity change that might be caused by a derangement of calcium metabolism. In my previous study (see Chapter III) I found that maximum ATP-independent calcium uptake in the presence of 1 mM calcium by the plasma membrane fraction from SHR arteries was significantly lower than that of NWR arteries. Furthermore, Hansen and Bohr (1975) have suggested that a decreased number of calcium binding sites in the plasma membrane of SHR may cause the increased excitability associated with hypertension of SHR. Therefore, in the light of their results, it will be of great interest to determine if the previously reported alteration in passive calcium uptake (see Chapter III) is present in pre-hypertensive SHR.

Clearly, the present investigation does not exclude the possibility that a primary change in calcium regulation is responsible for the alterations of reactivity observed in the SHR mesenteric artery. It may be that a derangement in calcium regulation exists but is not detected by the techniques employed. In addition to alterations in passive uptake, there may be changes in the rate of calcium uptake or release by membranes of SHR; this was not studied presently but further work will determine if such changes occur and if they can be correlated with alterations in phosphatase metabolism and with the time course of the development of hypertension.

E. Summary

Spontaneously hypertensive rats (SHR) and two strains of normotensive rats were compared with respect to enzymatic activities and calcium uptake of plasma membrane- and endoplasmic reticulum-enriched fractions from their mesenteric arteries. Increased specific activities of alkaline phosphatase, 5'-nucleotidase and Mg^{++}-ATPase, and increased ATP-dependent calcium uptake was found in 5-6 month old SHR as compared to both strains of age-matched normotensive rats. Alkaline phosphatase was increased in 33 day old "pre-hypertensive" and 3-4 month old SHR, but 5'-nucleotidase, Mg^{++}-ATPase and calcium uptake were not. Hydralazine treatment of young SHR partially prevented the increase of both alkaline phosphatase activity and blood pressure that develops with age. The relationship between alkaline phosphatase activity and the alterations in vascular reactivity associated with hypertension remains to be determined.

CHAPTER VI

CALCIUM UPTAKE AND ENZYMATIC ACTIVITIES OF SUBCELLULAR FRACTIONS FROM AORTAE AND VENTRICLES OF SPONTANEOUSLY HYPERTENSIVE RATS

CHAPTER VI: CALCIUM UPTAKE AND ENZYMATIC ACTIVITIES OF SUBCELLULAR FRACTIONS FROM AORTAE AND VENTRICLES OF SPONTANEOUSLY HYPERTENSIVE RATS

A. Introduction

Many changes have been reported in the reactivity of vascular smooth muscle in hypertensive animals and man, but little information is available concerning the possible underlying biochemical mechanisms (Folkow, 1971; Pickering, 1968; Somlyo and Somlyo, 1970). Since intracellular calcium activity is an important determinant of smooth muscle contractile state, an alteration in calcium regulation is a plausible cause of supersensitivity to excitatory agents of strips of arteries from hypertensive animals (Hinke, 1966; Field et al., 1972; Somlyo and Somlyo, 1970). Indeed it has been suggested that increased sensitivity to potassium and noradrenaline, and decreased rate of relaxation of aorta from 6-month-old spontaneously hypertensive rats (SHR) may be due to decreased calcium extrusion by the cell membrane (Field et al., 1972). In the present investigation this hypothesis was tested by isolating subcellular fractions from these aortae and studying their calcium transporting properties. Although the aorta is not a resistance vessel, there is some evidence that similar changes in ionic composition and weight occur in both large and small vessels of hypertensive animals (Jones, 1973; Somlyo and Somlyo, 1970) and alterations in contractile reactivity have been reported for both mesenteric (Haeusler and Haefely, 1970; 1972) and femoral artery (Holloway and Bohr, 1973) as well as aorta (Field et al., 1972; Shibata et al., 1973) from SHR.

Cardiac muscle of SHR also exhibits several changes in contractile activity and morphology associated with the development of hypertension (Farmer et al., 1974; Fujiwara et al., 1972) and sarcoplasmic reticulum from hypertrophied hearts have been reported to exhibit decreased calcium binding (Schwartz et al., 1973). Furthermore, Shibata and co-workers (Fujiwara et al., 1972; Shibata et al., 1973) have suggested that alterations of contractile responses of both heart and aorta of SHR are due to inherent differences in the musculature rather than to secondary changes caused by hypertension. Therefore, the calcium uptake characteristics of subcellular fractions from hearts of SHR and normotensive rats were also compared.

B. Methods and Materials

Animals and Tissue Preparations

Male spontaneously hypertensive Wistar rats of the Okamoto and Aoki strain (Okamoto and Aoki, 1963) and Wistar normotensive rats were 5-6 months of age and received a standard diet and tap water. Systolic blood pressure was recorded from the tail of pre-warmed, unanesthetized rats by tail plethysmograph using an electrosphygmomanometer and a Physiograph Four-A (E and M Instrument Co., Houston). Animals were killed by a blow on the head and their beating hearts and aortae were rapidly removed and placed in ice-cold 8% sucrose, 40 mM histidine buffer at pH 7.0 (the pH was adjusted with HCl; this buffer was used for all procedures unless otherwise stated). Ventricles were freed of blood clots and atrial tissue and sliced into 1-3 mm sections with scissors; these slices were washed several times with buffer to remove blood. All operations were carried out at 4°C unless otherwise stated.

The methods used for the isolation of plasma membrane enriched fractions are similar to those previously developed in this laboratory for isolating these fractions from rat heart and myometrium (Kidwai et al., 1971a; Kidwai et al., 1971b). Ventricle slices (3g) were homogenized in 6 volumes of buffer using a Polytron PT20 (Kinematic Gmbh) for 5 s (repeated once) at 15,000 rpm. The homogenate was centrifuged at 1,000 X *g* (Av) for 10 min to remove whole cells, nuclei, connective tissue and contractile elements; this step yields the 1,000 X *g* supernatant which was the starting material for both the mitochondrial and density gradient fractions. Mitochondria were prepared from ventricles by centrifuging the above supernatant for

10 min at 10,000 X *g* (Av) to yield the crude mitochondrial pellet and the 10,000 X *g* supernatant. The pellet was gently homogenized in sucrose-histidine buffer and centrifuged at 1,000 X *g* (Av) for 10 min. The supernatant was centrifuged at 10,000 X *g* (Av) for 10 min and the pellet was washed and resuspended in buffer; this centrifugation was repeated and the final pellet was resuspended in buffer to yield a uniform suspension containing 0.4-1 mg protein/ml. Teflon-glass homogenizers were used for suspending all pellets.

The supernatant from the first 10,000 X *g* centrifugation was spun at 113,000 X *g* (Av) for 30 min to yield a crude microsomal fraction which was suspended in 3 ml of 8% sucrose-40 mM histidine buffer and carefully laid on a sucrose gradient containing (from bottom to top), 4 ml of 45.0% sucrose and 3 ml each of 33.0% and 28.0% sucrose. The sucrose concentrations were measured with a refractometer. After centrifugation at 112,000 X *g* (Av) for 120 min., protein bands were carefully removed from the gradient tube using Pasteur pipettes. These bands were diluted with buffer and deionized distilled water to yield a final sucrose concentration of 8%. The suspension was centrifuged at 102,000 X *g* (Av) for 30 min to yield the final pellets which were used to prepare uniform suspensions of 0.2-1 mg protein/ml. Cardiac sarcoplasmic reticulum was prepared by homogenizing ventricle muscle in 10 mM sodium bicarbonate, 5 mM sodium azide buffer as described by Entman and co-workers (Entman et al., 1973), except that the final pellet was suspended in sucrose-histidine buffer. The method includes an extraction with 0.6 M KCl.

A plasma membrane enriched fraction from aorta was isolated by a procedure similar to that used above for isolating this fraction from ventricle. Aorta from twelve rats were cleaned of loosely bound fat and connective tissue and homogenized (1.2-1.4 g of tissue) in 25 ml of sucrose-histidine buffer for 10 s (twice) at 15,000 rpm using a Polytron PT 20. The remainder of the procedure (including the sucrose gradient used) was the same as described above for ventricle fractions except that the last 10,000 X *g*/10 min centrifugation was omitted. Attempts to obtain mitochondria by the procedure used for ventricle did not result in a significant yield of protein.

Enzymatic Determinations and Electron Microscopy

Phosphodiesterase I (Touster et al., 1970), 5'-nucleotidase, cytochrome c oxidase, phosphate and protein were determined as previously described (Kidwai et al., 1971b). Ouabain-sensitive, K^+-stimulated phosphatase was assayed (Kidwai et al., 1971b) using pH 7.4 and 1 mM ouabain. ATPases were determined under the same conditions as calcium accumulation in the presence of 1 μM free calcium (including the same EGTA concentration) except that calcium was omitted in measuring Mg^{++} - ATPase. This resulted in a free Ca^{++} concentration of less than 0.01 μM. Ca^{++} - ATPase is (Ca^{++} + Mg^{++})-ATPase minus Mg^{++} - ATPase.

Pellets were fixed in 2.5% glutaraldehyde in phosphate buffer (pH 7.1), post-fixed in OsO_4 and embedded in epon. Sections were stained with uranyl acetate and lead citrate, and photographed on a JEM 7A electron microscope.

Measurement of Calcium Uptake

Calcium uptake was measured by the millipore filtration technique (Martonosi and Feretos, 1964) using ^{45}Ca and liquid scintillation counting. The reaction mixture contained: 100 mM KCl, 5 mM $MgCl_2$, 0.1 mM $CaCl_2$, labelled with 0.4 μCi/ml ^{45}Ca, 40 mM imidazole (to buffer the solution to pH 7.0, and 10-50 μg of protein per final ml of reaction mixture (depending on the fraction used and other additions to the reaction mixture). Where indicated in the text, 5 mM disodium ATP, sodium azide or potassium oxalate were added. Variable amounts of EGTA were added to obtain calcium concentrations less than 17 μM. Calculation of the amount of EGTA to be added was according to the equations (Katz et al., 1970) and association constants (Godt, 1974) previously published. Calcium uptake was always studied simultaneously on freshly prepared fractions from both types of rat. Prior to use, all filters (25 mm, pore size: 0.45 microns; Matheson-Higgins Co. Inc.) were washed with 10 ml of 100 mM KCl solution followed by 10 ml of sucrose-imidazole (pH 7.0); this procedure resulted in low background counts. The reaction was started by adding protein to the reaction mixture (which had been preincubated for 5 min at 37°C), and stopped by filtration; this was followed by a wash with 10 ml of 8% sucrose-40 mM imidazole. The reaction time was 10 min unless otherwise indicated. At this time, calcium uptake was either increasing or at a plateau level. Filters were dissolved in 10 ml of Bray's solution (Bray, 1960) and counted to at least 2% accuracy.

Materials

Solutions were prepared from distilled deionized water and analytical grade reagents. Organic compounds were the highest purity available from Sigma; $^{45}CaCl_2$ was obtained from Amersham-Searle.

Dr. R. Hamill of Eli Lilly and Dr. J. Berger of Hoffman-LaRoche generously supplied A23187 and X-537A, respectively. These calcium ionophores were dissolved in ethanol and added to the 1 ml reaction mixture in a volume of 10 µl.

Data Analysis

Values were compared by the Student's *t*-test and differences with P values of 0.05 or less were considered significant. Standard errors of the means are bracketed in the figures except where the standard error is less than the symbol size, or where omitted for clarity.

C. Results

Enzymatic Determinations and Electron Microscopy

The mean systolic blood pressure of the spontaneously hypertensive rats used in this study was 190 ± 7.0 mm Hg (N = 36) vs. 125 ± 5.5 mm Hg for normal Wistar rats (N = 36). The enzymatic activities of the fractions from rat ventricles are shown in Table 1. The fractions designated plasma membrane were the lightest fractions from the gradient (at the 8%/28% interface) and exhibited the highest specific activity of plasma membrane marker enzymes (5'-nucleotidase; ouabain-sensitive, K^+-stimulated phosphatase and phosphodiesterase I). They had the lowest specific activity of cytochrome c oxidase; this was only 3.6% of the specific activity of oxidase in the mitochondrial-rich band at the 33%/45% interface obtained in 6 experiments omitting the first 10,000 X *g* centrifugation of the preparation. Subfractionation of the band at the 8%/28% interface using a gradient of 22% to 28% bands of sucrose did not yield any fractions of significantly higher specific activity of plasma membrane marker enzymes than that from the original. Electron micrographs of the plasma membrane fraction were essentially the same as those previously published (Kidwai et al., 1971a) for this fraction isolated by a similar procedure, and revealed the presence of membrane vesicles but no mitochondria or rough endoplasmic reticulum.

All denser fractions (e.g. 28%/33%) which exhibited lower activity of 5'-nucleotidase and ouabain-sensitive, K^+-stimulated phosphatase were contaminated with broken mitochondria. Cardiac sarcoplasmic reticulum enriched membrane was, therefore, prepared by differential centrifugation only (Entman et al., 1973). This fraction, designated sarcoplasmic reticulum in Table 1 contained relatively low plasma membrane contamination (compared with all gradient fractions which, like this material, were low in mitochondrial contamination).

The fraction designated mitochondrial in Table 1 was isolated by differential centrifugation and exhibited even lower specific activities of plasma membrane enzymes than did the sarcoplasmic reticulum fraction (Table 1). The only significant differences between the enzyme activities of fractions from normotensive and hypertensive rats was the greater ouabain-sensitive, K^+-stimulated phosphatase (Table 1) of the hypertensive plasma membrane fraction.

The lightest protein band (8%/28% interface) from the sucrose gradient of aortae was found to exhibit the highest specific activity of plasma membrane marker enzymes (Table 2). This fraction was designated plasma membrane. The protein band from the 28%/33% sucrose interface contained less plasma membrane than the 8%/28% interface fraction (Table 2). It was designated endoplasmic reticulum (ER) since relative to the lighter fraction it was enriched in this membrane. There was a 2.8-fold (range from 1.7 to 4.2, n = 6) increase in ATP-dependent calcium uptake in the presence of oxalate during a 10 min incubation period in this fraction, but no significant increase was obtained in the fraction designated as plasma membrane under the same conditions. This "oxalate effect" is characteristic of the endoplasmic reticulum, and helps differentiate the two fractions on this parameter in vascular smooth muscle. Electron micrographs of these fractions were essentially the same as those published for the corresponding fractions from myometrium (Kidwai et al., 1971b) except that pieces of collagen were occasionally seen in the aorta fractions.

TABLE 1

Enzymatic Activities of Fractions from Rat Ventricles

ENZYME AND TYPE OF RAT	FRACTION (Origin from Gradient or Otherwise)			
	1000 X *g* Supernatant	Plasma Membrane (8%/28%)	Sarcoplasmic Reticulum (Centrifugation)	Mito-chondria (Centrifugation)
5'-Nucleotidase				
Normotensive	1.49±0.29 (4)	19.5 ±1.41 (9)	5.4±1.20 (4)	3.50±0.91 (5)
Hypertensive	1.26±0.26 (4)	19.9 ±1.82 (7)	4.4±0.87 (4)	2.79±0.87 (6)
Phosphodiesterase I				
Normotensive	0.46±0.04 (5)	5.6 ±0.3 (7)	2.4±0.4 (5)	0.67±0.07 (4)
Hypertensive	0.42±0.05 (5)	4.9 ±0.5 (7)	2.2±0.3 (5)	0.53±0.08 (5)
Ouabain-sensitive, K^{+}-stimulated phosphatase				
Normotensive	0.16±0.13 (8)	2.01±0.35 (8)	0.80±0.15 (7)	0.10±0.16 (5)
Hypertensive	0.17±0.10 (8)	*2.75±0.21 (8)	1.13±0.10 (7)	0.15±0.04 (4)
Mg^{++}-ATPase				
Normotensive	36.0 ±3.0 (7)	283±7.1 (7)	75.8 ±5.2 (7)	-
Hypertensive	32.2 ±2.8 (7)	294±6.4 (7)	74.5 ±4.3 (7)	-
Ca^{++}-ATPase				
Normotensive	3.9 ±0.3 (7)	36.4 ±2.5 (7)	9.2 ±0.6 (7)	-
Hypertensive	4.1 ±0.2 (7)	34.5 ±2.1 (7)	8.8 ±0.5 (7)	-

Specific activities are expressed as μmoles phosphate released per mg protein per hour for 5'-nucleotidase, Mg^{++}-ATPase and Ca^{++}-ATPase, and as μmoles p-nitrophenol released per mg protein per hour for phosphodiesterase I and ouabain-sensitive K^{+}-stimulated phosphatase. Numbers in parentheses are the number of independent preparations tested.

*Significantly different from normotensive value.

TABLE 2

Enzymatic Activities of Fractions from Rat Aortae

ENZYME AND TYPE OF RAT	FRACTION (Origin from Gradient)		
	1000 X *g* Supernatant	Plasma Membrane (8%/28%) (Interface)	Endoplasmic Reticulum (28%/33%) (Interface)
5'-Nucleotidase			
Normotensive	7.1 ± 1.0 (6)	30.4 ± 3.9 (12)	20.8 ± 3.4 (10)
Hypertensive	11.1 ± 1.4 (6)	*55.6 ± 5.1 (12)	29.1 ± 2.0 (8)
Phosphodiesterase I			
Normotensive	0.50± 0.04 (5)	2.81± 0.17 (5)	1.23± 0.51 (5)
Hypertensive	0.48± 0.11 (5)	*3.96± 0.30 (5)	1.01± 0.15 (5)
Ouabain-sensitive, K^+-stimulated phosphatase			
Normotensive	0.13± 0.06 (6)	1.90± 0.27 (6)	0.49± 0.11 (6)
Hypertensive	0.18± 0.05 (6)	1.76± 0.18 (6)	0.55± 0.13 (6)
Mg^{++}-ATPase			
Normotensive	106.3 ±10.2 (6)	633 ±14.5 (6)	526 ±12.7 (6)
Hypertensive	92.4 ± 8.6 (6)	595 ±20.4 (6)	554 ±28.0 (6)

Values are expressed as in Table 1.

*Significantly different from normotensive.

The yield of protein in the 1,000 X *g* supernatant from 12 rat aortae (1.2-1.4 g tissue) was 10.5 ± 1.7 and 10.7 ± 1.8 mg (N = 8 each) for normotensive and hypertensive rats, respectively. The plasma membrane and ER fractions contained only 2.7% and 1.4% respectively of this protein. The protein yield of these fractions could be doubled by increasing the homogenization time to 40 s, but this resulted in a marked decrease in the specific activity of plasma membrane marker enzymes. Although the yield of these fractions was the same for both hypertensive and normotensive rats, the specific activities of their enzymes differed greatly (Table 2). Phosphodiesterase I and 5'-nucleotidase activities were increased in the plasma membrane fraction from hypertensive rats while Mg^{++}-ATPase and ouabain-sensitive, K^{+}-stimulated phosphatase activities were similar to those from normotensive rats. No significant Ca^{++}-ATPase activity (in the presence of 1 μM or 17 μM free calcium) was detected in these fractions; the very high Mg^{++}-ATPase activity would tend to mask any reasonable amount of Ca^{++}-ATPase.

Calcium Uptake Studies

Calcium uptake was increased in all ventricle fractions by the addition of ATP to the reaction mixture but there was no difference between hypertensive and normotensive membranes in this regard (Table 3). Plasma membrane and sarcoplasmic reticulum fractions exhibited a large increase in calcium uptake in the presence of oxalate but no inhibition by azide, while uptake by mitochondria were affected by azide but not oxalate. Subfractionation of the plasma membrane preparations using a gradient of 22% to 28% sucrose did not result in

TABLE 3

Calcium Uptake by Fractions from Rat Ventricles

ADDITION TO REACTION MIXTURE AND TYPE OF RAT	FRACTION Plasma Membrane	Sarcoplasmic Reticulum	Mito-chondrial
None			
Normotensive	1.12± 0.59	1.50± 0.38	0.51± 0.15
Hypertensive	1.25± 0.11	1.67± 0.47	0.43± 0.22
5 mM ATP			
Normotensive	27.8 ± 3.9	14.5 ± 2.4	267 ±43
Hypertensive	25.9 ± 3.1	15.8 ± 1.9	235 ±72
5 mM ATP plus 5 mM azide			
Normotensive	22.4 ± 4.1	14.0 ± 2.7	0.72± 0.14
Hypertensive	25.0 ± 1.8	15.2 ± 2.1	0.52± 0.36
5 mM ATP plus 5 mM oxalate			
Normotensive	674 ±85	310 ±35	208 ±55
Hypertensive	604 ±29	295 ±27	190 ±58

Values are expressed as μmoles Ca^{++} taken up g^{-1} protein during an incubation of 10 minutes at 37°C. The reaction mixture contained 17 μM free Ca^{++}, 5 mM $MgCl_2$, 100 mM KCl and 40 mM imidazole at pH 7.0. N = 6.

any fraction which exhibited increased plasma membrane marker enzyme activity and decreased oxalate stimulation, suggesting that ATP-dependent calcium transport is a property of rat ventricle plasma membrane. However, the lack of an unequivocal marker enzyme for cardiac sarcoplasmic reticulum makes it impossible to estimate the contribution by this membrane to the calcium uptake of this fraction.

A study of the dependence of calcium uptake on the calcium concentration (Fig. 1) also failed to reveal a difference between the hypertensive and normotensive heart fractions. Other fractions from the sucrose gradient (at the 28% and 33% interface and within the 33% sucrose band) which were believed to be a mixture of plasma membrane and sarcoplasmic reticulum, also exhibited no difference between hypertensive and control fractions (not shown). Similarly there was no apparent difference between these fractions in the rate of calcium uptake as estimated with a 30 s incubation time; for example, the hypertensive plasma membrane fraction bound 2.5 ± 1.3 μmoles/g while the normotensive 3.6 ± 1.1 μmoles calcium/g protein (N = 5 for each).

In contrast to heart fractions, aortic preparations showed a decreased calcium uptake by the plasma membrane enriched fraction from spontaneously hypertensive compared to that from normotensive rats (Table 4, Fig. 2). The lack of inhibition by azide indicates that this calcium uptake is not attributable to mitochondria (Table 4). No significant increase in calcium uptake was found in the presence of oxalate. However, the divalent ion ionophores, A23187 and X-537A markedly inhibited calcium uptake (Table 5). A study of the dependence of calcium uptake on free calcium concentration demonstrates that the

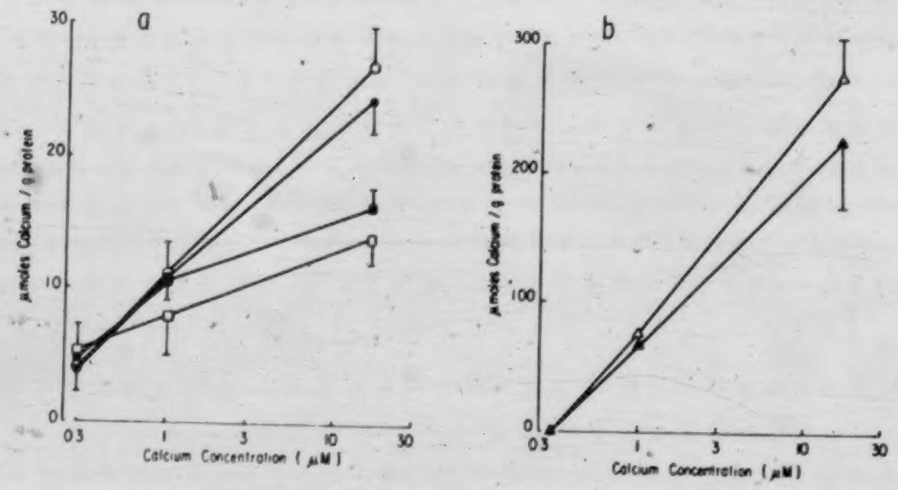

FIGURE 1. Effect of calcium concentration on calcium uptake by fractions from ventricles of normotensive (open symbols) and spontaneously hypertensive rats (closed symbols): (a) Circles, plasma membrane-enriched; squares, sarcoplasmic reticulum; (b) triangles, mitochondrial; N = 5-6. Calcium uptake was measured at the end of 10 min. after incubation in the presence of 5 mM ATP.

TABLE 4

Calcium Uptake by Fractions from Rat Aortae

ADDITION TO REACTION MIXTURE AND TYPE OF RAT	FRACTION		
	1000 X *g* Supernatant	Plasma Membrane	Endoplasmic Reticulum
None			
Normotensive	2.40 ± 0.23	5.40 ± 0.30	1.89 ± 0.53
Hypertensive	2.06 ± 0.12	5.01 ± 0.67	2.30 ± 0.40
5 mM ATP			
Normotensive	4.20 ± 0.45	16.2 ± 1.58	14.4 ± 1.60
Hypertensive	3.92 ± 0.70	*11.3 ± 0.39	11.76 ± 0.95
5 mM ATP plus 5 mM azide			
Normotensive	3.63 ± 0.30	16.1 ± 0.54	16.4 ± 3.00
Hypertensive	3.10 ± 0.05	*11.4 ± 1.12	12.5 ± 1.40

Values are expressed as in Table 3, and the reaction mixture is the same.

*Significantly different from normotensive.

N = 4, except for addition of ATP only, where N = 8.

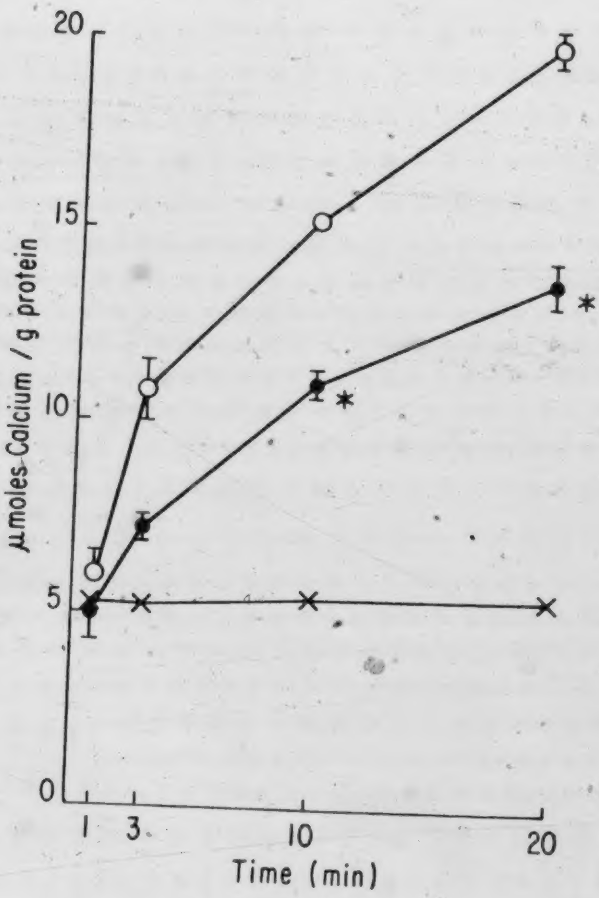

FIGURE 2. Time course of calcium uptake by plasma membrane-enriched fractions from aortae of normotensive (open circles) and spontaneously hypertensive rats (closed circles) in the presence of 5 mM ATP. Calcium uptake in the absence of ATP for this fraction from normotensive rats is also shown (x). The ATP-independent values for hypertensive rats (not shown) were not significantly different from those for normotensive rats (N = 4).

TABLE 5

Effect of Ionophores on Calcium Uptake by Fractions from Rat Aortae

Fraction and Type of Rat	Control	95 % Ethanol 1%	X537A 20 μM	A23187 10 μM
Plasma Membrane				
Normotensive	16.95±0.96	18.15±1.25	4.77±0.34	4.05±0.72
Hypertensive	11.80±0.92	11.83±0.90	4.58±0.31	4.50±0.29
Endoplasmic Reticulum				
Normotensive	14.23±0.61	12.93±1.34	3.68±0.27	3.45±0.53
Hypertensive	11.09±0.64	12.45±1.23	3.58±0.22	4.58±0.47

Values are expressed as in Table 3, and the reaction mixture is the same except that ethanol, or ionophores dissolved in ethanol, are added where indicated.

N = 4.

significant differences between hypertensive and normotensive fractions persisted at 1 μM free calcium (Fig. 3).

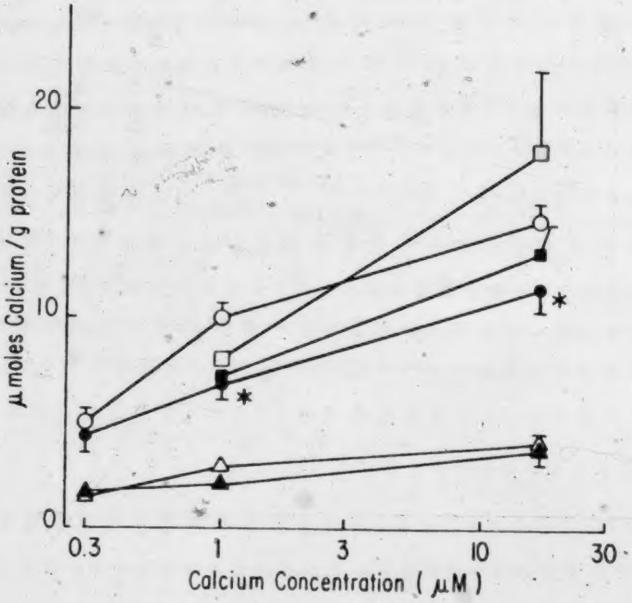

FIGURE 3. Effect of calcium concentration on calcium uptake by plasma membrane-enriched fractions (circles), endoplasmic reticulum-enriched fractions (squares), and the 1,000 X *g* supernatant (triangles) from aortae of normotensive (open symbols) and spontaneously hypertensive rats (closed symbols). Calcium uptake was measured at the end of 10 min. after incubation in the presence of 5 mM ATP. Asterisk indicates a significant difference from normotensive values (N = 5-8, except plasma membrane fraction at 17 μM free calcium, where N = 20).

D. Discussion

Enzymological and histochemical studies of arterioles, arteries and aortae of hypertensive animals report inc[illegible]MPase (Ichijima, 1969; Oka and Angrist, 1965) and increased alkaline phosphatase activities (Gardner and Laing, 1965; Ooshima, 1973). Therefore, the increased 5'-nucleotidase activities observed in the present study (Table 2) of plasma membrane enriched fractions from SHR aortae were to be expected. It is interesting to note that similar increases in these enzymes were not seen in subcellular fractions from SHR heart (Table 1), but were obtained in membrane fractions from mesenteric arteries (see Chapter III). The problem of interpreting results in studies of this type without knowing which strain of rat is the best control has been previously discussed (Lais et al., 1974), however, the similarity between my results and those of other workers (cited above) using different normotensive strains as well as different methodology suggest that the increased specific activities of phosphodiesterase I as well as 5'-nucleotidase, represent real abnormalities associated with hypertension. I have found similar differences in the hypertensive arteries from those of two other normotensive strains (see Chapter V). The co-purification of these plasma membrane marker enzymes (DePierre, 1973) with ouabain-sensitive K^+-phosphatase (Table 2) suggests that the increases in AMPase activities observed in histological studies (Ichijima, 1969; Oka and Angrist, 1965) are localized, in part, at the plasma membrane. However, the lack of a proven marker enzyme for endoplasmic reticulum of vascular smooth muscle makes it impossible to estimate the amount of these intracellular membranes in the plasma membrane enriched fractions.

Previous investigators have reported that the earliest change found in hypertrophied and failing hearts is a decrease in the rate of calcium release, followed by a decreased rate of uptake and a decreased total amount of calcium taken up (Schwartz et al., 1973). Under the conditions of my experiments, little difference was seen between the calcium uptake characteristics (Table 3, Fig. 1) of the various fractions studied. After I completed my study, Aoki and co-workers (Aoki et al., 1974) reported a small but significantly lower maximum calcium uptake capacity of microsomes from SHR ventricles compared with normotensive (8.80 ± 0.33 vs. 11.95 ± 0.55 μmoles/g, N = 8-10). Their microsomes were prepared from younger rats than used in my study, and calcium uptake was determined in a reaction mixture different from mine. In sarcoplasmic reticulum fractions from SHR and normotensive rat ventricles at 3 months of age in which uptake of calcium at pH 6.4 was determined, I found no significant differences between the two groups (17.8 ± 1.8 vs. 18.1 ± 2.1 μmoles/g protein/10 min, N = 4).

The increased specific activities of 5'-nucleotidase and phosphodiesterase I in the plasma membrane enriched fraction from SHR aortae (Table 2) suggests that the lower calcium uptake of this fraction (Table 4) is not the result of a greater contamination with non-membranal protein. Furthermore, inhibition of ATP-dependent calcium uptake by the ionophores X-537A and A23187 (Table 5) suggests that calcium is being transported across the vesicle membrane (Scarpa et al., 1972), since these ionophores should prevent development of a gradient of calcium across the membrane. However, there is some evidence that these ionophores may also act by inhibiting calcium binding (Entman et al., 1973). Presumably these vesicles are composed of "inside-out" plasma membrane, as are those of erythrocytes (Weiner and Lee, 1972) which also exhibit ATP-dependent calcium transport. It should be noted that the magnitude of this calcium uptake is similar to that reported for microsomal fractions from rabbit (Fitzpatrick et al., 1972) and bovine aorta (Hess and Ford, 1974). Since the completion of my study, Aoki and co-workers (1974) have reported that SHR microsomes prepared from vascular smooth muscle pooled from aorta and several arteries also exhibited a reduced calcium uptake capacity when compared with that prepared from normotensive rat.

A decreased ATP-dependent calcium transport of the SHR aortic membranes is consistent with the hypothesis that the slower rate of relaxation of the SHR aortae (Field et al., 1972) is due to a decrease in the rate of ATP-dependent calcium efflux from the smooth muscle cell. It is not known if the increased sensitivity of aortae from 6-month-old SHR to norepinephrine and potassium chloride is related to their slower rate of relaxation after contraction induced by potassium chloride (Field et al., 1972), but studies with aortae from renal hypertensive rats demonstrate that changes in relaxation rates are seen as soon as hypertension (b.p. 140-150 mm Hg) and vascular supersensitivity to potassium chloride develop (Field et al., 1973). Additional studies will be required to determine if any causal relationships exist between the observed alterations in enzymatic and calcium uptake activities and hypertension.

There is some evidence suggesting that increased reactivity of vascular smooth muscle to excitatory agents cannot be explained entirely by either a decrease in the activity of processes that lower intracellular calcium activity or by an increase in the wall thickness to lumen size ratio (Folkow, 1971). For example, the change in sensitivity to potassium chloride of aortae from 6-month-old SHR is much greater than that to norepinephrine (Field et al., 1972), and the change in sensitivity of perfused vasculature of SHR is greater to serotonin than to norepinephrine (Haeusler and Finch, 1972). These results indicated that there are specific changes in membrane excitability that allow for differential changes in sensitivity to various excitatory agents; these alterations are in addition to any changes in calcium transport and in vascular geometry that would tend to produce a similar degree of hyper-reactivity to all excitatory agents.

E. Summary

Subcellular fractions were obtained from aortae and ventricles of 6-month-old spontaneously hypertensive and normotensive Wistar rats by the use of differential and sucrose density gradient centrifugation. These preparations were studied to determine what alterations in calcium uptake and enzymatic activities might be associated with hypertension. The total amount of calcium uptake (in the presence of ATP and 17 μM free calcium) by the plasma membrane enriched fraction from hypertensive rat aortae was significantly less than that from normotensive rats (11.3 ± 0.4 vs. 16.2 ± 1.6 μmole calcium/g protein, n = 8). In contrast the specific activities of the plasma membrane marker enzymes, 5'-nucleotidase and phosphodiesterase I, were 80% and 40% greater, respectively, in the hypertensive than in the normotensive fractions. On the other hand, various fractions from ventricles of the two types of rats were generally similar in enzyme activities and calcium uptake. The decreased rate of relaxation of aortae from spontaneously hypertensive rats may be caused by the decreased rate of calcium transport observed in this study.

CHAPTER VII

GENERAL DISCUSSION

CHAPTER VII: GENERAL DISCUSSION

A. Calcium Sequestration System in Subcellular Membranes of the Cardiovascular System

The intracellular calcium distribution of cells must be carefully controlled because calcium plays a key regulatory role in a variety of cellular processes (Rasmussen, 1970; Triggle, 1972; Lehninger, 1974). For excitation-contraction coupling of striated muscle, there is general agreement that sarcoplasmic reticulum is the major regulator of calcium activity (Hasselbach and Makinose, 1962; Sandow, 1965; Weber, 1966), but there is some dispute as to the relative regulatory contributions of sacroplasmic reticulum and mitochondria in cardiac muscle (Lehninger, 1974; Solaro and Briggs, 1974; Katz et al., 1966). Since both sarcolemma and mitochondria from these muscles are able to transport calcium, a role for these organelles in lowering intracellular calcium following muscle contraction has been suggested (Sulakhe et al., 1973; Dransfeld et al., 1969; Fanburg and Gergely, 1965; Sordahl et al., 1973).

At the present time, the relative importance of these subcellular organelles in regulation of intracellular calcium in vascular smooth muscle is unclear, although microsomal fractions isolated from large vessels have been shown to transport calcium (Hess and Ford, 1974; Fitzpatrick et al., 1972; Baudouin and Meyer, 1973). It is known that the endoplasmic reticulum occupies a small amount of cell volume in guinea pig mesenteric arteries (1.8%), rabbit aorta (5%) (Devine et al., 1972) and rat ventricle (3.5%) (Page et al., 1971), as compared to the volume occupied in the frog sartorius (13%) (Peachey, 1965). It is also known that the dependency of contraction on extracellular calcium differs between types of smooth muscles (Devine et al., 1972). It has been proposed that mitochondria may contribute to the regulation of intracellular divalent cation levels in smooth muscle based on studies of strontium or barium accumulation by electron probe analysis (Somlyo et al., 1974).

All the above statements lead one to question whether the endoplasmic reticulum from vascular smooth muscle plays the major role in the regulation of intracellular calcium. In this study, I succeeded in isolating subcellular fractions enriched in plasma membrane and other fractions probably enriched in endoplasmic reticulum, which allowed me to further investigate the ability of these fractions to remove calcium from solutions of physiological calcium concentrations. If one assumes that the calcium uptake capacity of each fraction in the presence of ATP (i.e. steady state uptake value times total amount of protein) is an indicator of its regulatory ability, the results of this study suggest that the capacity of plasma membrane is larger than that of endoplasmic reticulum from cardiovascular system. The results of this study also reveals that vascular smooth muscle mitochondria can accumulate calcium in the presence of ATP. Since the completion of my work, two other reports on calcium uptake by vascular smooth muscle mitochondria have appeared (Vallieres et al., 1975; Ford and Hess, 1975). This property is similar to that reported for mitochondria isolated from skeletal and cardiac muscle (Fanburg and Gergely, 1965; Brierly et al.,1964; Samaha and Gergely, 1965). Therefore, it is also possible that the mitochondria play a role in vascular smooth muscle contractility.

However, complete understanding of the relationship of this type of calcium uptake study to the calcium transport during excitation-contraction process in intact cells is far from being achieved as explained below:

Unfortunately, several factors are unknown about the systems in this study, those of major importance being the intracellular pH and the intracellular concentration of Mg^{++} and ATP and hence Mg-ATP. The ability of the various fractions to transport calcium is undoubtedly dependent upon these three variables, and Godt (1974) had clearly shown that the Ca^{++} dependence of the activity of skinned frog muscle fibers is dependent upon the Mg-ATP concentration. In addition, it has also been suggested that functional relationships might exist in smooth muscle between closely associated plasma membrane, endoplasmic reticulum and mitochondria (Devine et al., 1973; Goodford and Wolowyk, 1972). Such relationships are lost in subfractionation studies of this type.

Therefore, a conservative conclusion is drawn at this stage: That is, in cardiovascular tissue, in addition to endoplasmic reticulum, plasma membrane and mitochondria may play important roles to lower free calcium concentration after muscle contraction.

B) Calcium Uptake and Enzymatic Activities in the Cardiovascular System of SHR

In order to understand the pathology and etiology of hypertension, it is important to have detailed information on the biochemical changes of the whole cardiovascular system. In this study, the specific activities of many enzymes and the rate of calcium uptake were studied in the subcellular fractions from small and large vessels, and ventricles from SHR. Most of the enzymes studied: alkaline phosphatase, 5'-nucleotides, K^{+}-activated phosphatase, phosphodiesterase I are generally believed to be located mainly in plasma membrane.

In the studies on the plasma membrane fraction of cardiovascular systems from normotensive rats and SHR, I found many differences between these types of rats, when fractions from arteries of age-matched rats were compared. The specific activities of all the above mentioned enzymes as well as Mg^{++}-ATPase were greater from mesenteric arteries of SHR; 5'-nucleotidase and phosphodiesterase I were greater from aortae of SHR; whereas only ouabain sensitive K^{+}-phosphatase was higher from ventricles of SHR. Active calcium uptake was greater and passive calcium uptake was less from plasma membrane fractions of mesenteric arteries of SHR, and active calcium uptake of plasma membrane from aortae of SHR was lower. No changes in calcium uptake from membrane fractions of ventricles of SHR were detected compared to similar values from NWR rats. Thus, more parameters were altered in vascular tissue, especially in small mesenteric arteries, than in cardiac muscle of hypertensive rats. This fact is consistent with the view that elevated blood pressure in well-developed hypertension either causes or is the result of pathological change in small arteries.

It is well documented (Somlyo and Somlyo, 1970; Weiss, 1974) that increased total peripheral resistance in hypertension is related to the altered vascular reactivity. "Vascular reactivity" is usually measured as the magnitude of an increase in flow resistance or an increase in pressure produced by a constrictor agent. The magnitude of this increase could depend either on an increase in sensitivity of the vascular smooth muscle or on a thickening of the vessel wall. There is evidence in the literature to support each of these possibilities, accompanied by controversy between the champions of the two mechanisms as to which is mainly responsible for the increased vascular reactivity that is seen in hypertension.

Recently Folkow (1971) has summarized his evidence that most, if not all, of the increase in vascular reactivity in rats with genetic hypertension and in man with essential hypertension is caused by an increase in wall thickness. Concentration-response curves of vascular resistance to norepinephrine for hypertensive animals differed from those from normotensive animals in the same way as did the calculated concentration-response curves of a mathematical model in which it was assumed that medial thickness had increased by 30% and the increase in wall thickness had encroached on the lumen when the smooth muscle was completely relaxed. No change in the threshold dose for norepinephrine was found by Folkow. The failure of Folkow to obtain an increased sensitivity to norepinephrine may be due to his use of an artificial medium for perfusion or to papaverine treatment (see Lais and Brody, 1975).

In this study, an increase of specific activity of 5'-nucleotidase in hypertensive arteries might support Folkow's view, since increased 5'-nucleotidase (AMPase) activity was reported in proliferating muscle cells of arterial walls (Oka and Angrist, 1967). However, an increase of specific activities of other enzymes, which appear to have no known functional role in proliferation or in the increase of wall thickness, obviously cannot be explained simply by this hypothesis.

Furthermore, according to Johansson's analysis (1974), vasoconstriction of smooth muscle would be most sensitive to changes in the particular factor (Ca^{++} or ATP) that is normally the rate limiting determinant of the chemomechanical transduction, and therefore one might predict that this factor would be altered and therefore causes a change in reactivity. The alterations of calcium regulation in vascular smooth muscle of SHR observed in this study are consistent with this analysis. Such a change certainly cannot be explained by Folkow's hypothesis. Therefore, the suggestion is made that changes in both calcium metabolism and vessel wall thickness may be important for the development of vascular hyperreactivity.

All the above statements indicate that these changes may be related to hypertension. Therefore, it is of interest to know which parameter(s) is more intimately related to the initiating cause or effect of hypertension. Insight on this question can be obtained from the experiments on young (33 days) and initial hypertensive (3-4 months) rats, and on hydralazine treated NWR and SHR. Slight differences in the above mentioned parameters, except alkaline phosphatase, were found in subcellular fractions from arteries of young SHR as compared to those from NWR. Thus the elevation of blood pressure preceded the changes in activities of most enzymes studies and the changes in Ca^{++} uptake. The treatment of NWR and SHR with hydralazine decreased the elevation in blood pressure with age, and also decreased the increase in specific activity of alkaline phosphatase with age. Hence the conclusion is drawn that alkaline phosphatase activity may be closely associated with the initial cause of hypertension, and that changes of other parameters may be the result of hypertension or related to development of chronic hypertension. However, the 33 days old rats did have a significantly elevated blood pressure which might have caused the increased alkaline phosphatase activity. The physiological functions of alkaline phosphatase are not clear, but Wyke and Gardner (1970) suggested it may be related to vascular permeability. However, it would be of considerable interest to know if the phosphatase that I studied in SHR membranes has protein phosphatase activity.

There is increasing evidence that phosphoproteins may play a key role in regulating Ca^{++} transport and ATPase activity as well as membrane permeability (Rubin and Rosen, 1975; Tada et al., 1975), and it is known that some alkaline phosphatases have protein phosphatase activity (Lee and Nickol, 1974). Furthermore, Sirica and co-workers (1975) have considered the possibility that decreased Mg^{++}-ATPase and 5'-nucleotidase, and increased alkaline phosphatase activities of regenerating liver plasma membrane may be associated with specific membrane transport abnormalities.

Many differences were found between the characteristics of aortae and mesenteric arteries in hypertension in this study. For example, calcium uptake by subcellular fractions from aortae (which requires more than 20 min to reach maximum) was slower than the calcium uptake by fraction from mesenteric arteries (which requires about 10 min to reach maximum). Furthermore, ATP-dependent calcium uptake by the plasma membrane fraction from SHR mesenteric arteries was significantly greater than that from NWR mesenteric arteries. In contrast, the ATP-dependent calcium uptake by the fraction from SHR aortae was decreased as compared to that from NWR vessels. These qualitative and quantitative differences might reflect a difference in the mechanisms controlling contraction and relaxation of large and small vessels. Therefore, aorta might not be as good a model for resistance vessels of hypertension than are mesenteric arteries. Also in support of this statement is the fact that increased sensitivity of aorta to KCl is seen only after several months of hypertension (Janis and Triggle, 1973).

C) Significance of the Data in Relation to Hypertension

In the following section, a brief discussion will be given on whether the results of the alterations of enzyme activities and calcium uptake in the plasma membrane vesicles from mesenteric arteries of 5-6 months old SHR can fit several simple hypotheses.

1. The first hypothesis to be considered is whether the essential difference between plasma membrane vesicles from arteries of normotensive and hypertensive rats is that more of the latter are inside-out type; i.e., those vesicles having the cytoplasmic face of their membrane on the outside.

Some of the results can fit this hypothesis, if one assumes, reasonably, that the active sites of Ca^{++}-ATPase and K^{+}-phosphatase are associated with the interior aspect of the plasma membrane. Since the hypothesis states that there are more inside-out type of plasma membrane vesicles from arteries of SHR, one would expect that more active sites of these enzymes to be exposed at the outer surface of the vesicles. If the Ca^{++}-ATPase is involved in Ca^{++}-transport, a greater ATP-dependent calcium uptake by plasma membrane from arteries of SHR would also be expected, since a greater proportion of such vesicles would develop a calcium gradient between inside and outside of the sealed vesicles as compared to vesicles from NWR. This difference of ATP-dependent calcium uptake between normotensive and hypertensive fractions should be obliterated by the presence of ionophores, if ionophores relax the calcium gradients across the vesicle membrane. This result was in fact observed. These findings are consistent with the hypothesis.

The greater specific activity of K^{+}-phosphatase in the plasma membrane fraction of SHR arteries as compared to that of NWR also can fit this hypothesis. However, the active sites of other enzymes, which had a higher specific activity in this fraction (such as 5'-nucleotidase, Mg^{++}-ATPase, leucine aminopeptidase, alkaline phosphatase) are probably associated with the exterior aspect of plasma membrane (Trams and Lauter, 1974). As the hypothesis states that more plasma membrane vesicles from arteries of SHR are inside-out, fewer of their active sites should be exposed at the exterior surface of the vesicles. The large, charged molecules of the substrates for these enzymes probably penetrate the vesicle membrane slowly, if at all; therefore, the increased specific activities of the above-mentioned enzymes in the plasma membrane of SHR arteries are not consistent with the hypothesis. However, one should realize that there are many uncertainties about the assumption made. For example, it is still possible that substrate-enzyme interaction can occur on either side of membrane vesicles from plasma membrane of smooth muscle.

If binding of Ca^{++} in the absence of ATP occurs mostly at the exterior surface of the plasma membrane the difference between plasma membrane fractions from SHR and NWR arteries in ATP-independent calcium uptake could be explained. Then the decreased Ca^{++}-uptake in plasma membrane fractions from SHR arteries would be expected. The ability of divalent cations to compete with $^{45}Ca^{++}$-uptake in the presence of ATP or in the absence of ATP by the plasma membrane fractions between NWR and SHR arteries may also be related to the different proportion of inside-out or rightside-out type membrane vesicles if one assumes that the two sides of the vesicles have different calcium binding or uptake sites. For example, Ca^{++} (1 mM) and Sr^{++} (10 mM) can displace ATP-dependent $^{45}Ca^{++}$-uptake by the plasma membrane fraction from SHR to a greater extent than that from NWR, whereas the amount displaced by Ba^{++} (1 mM) is considerably less. The uptake after 10 mM Sr^{++} probably represents non-specific binding of $^{45}Ca^{++}$ and most uptake after 1 mM Ca^{++} probably is by the same mechanism. The values for residual non-specific binding are the same for vesicles from SHR and NWR. Thus differences between the two types of vesicles in $^{45}Ca^{++}$-uptake represent differences in active binding or transport, and the greater effects of Sr^{++} and Ca^{++} on $^{45}Ca^{++}$-uptake for SHR vesicles would be expected from the greater active uptake when more vesicles are inside-out. My results would thus imply a differential accessibility of the transported or bound $^{45}Ca^{++}$ to competition from Ca^{++} or Sr^{++} on the one hand, and to Ba^{++} on the other hand. Conceivably Ba^{++} was unable to inhibit ATP-dependent binding or transport as effectively in vesicles of SHR arteries as in vesicles of NWR arteries; but this could not be explained by the presence of more inside-out vesicles in this case. Alternately, the $^{45}Ca^{++}$-uptake inhibited by Ba^{++} was that occuring non-specifically, or independent of ATP on the outside of vesicles. Then the greater displacement of $^{45}Ca^{++}$ by Ba^{++} in vesicles from NWR would be expected.

The results of cation interaction on ATP-independent $^{45}Ca^{++}$-uptake are difficult to interpret on the basis of more inside-out vesicles from SHR than from NWR. Ba^{++} was more effective in the former, thus contradicting the above explanation of its differential effect on ATP-dependent $^{45}Ca^{++}$-uptake. Moreover, to explain the effect of Mn^{++}, one would have to postulate that Mn^{++} affects mainly ATP-independent binding to right side-out vesicles. All-in-all these results are difficult to explain fully on the bases of this hypothesis. To further this hypothesis, the following experiments are suggested:

(a) Comparative study of enzymatic activities and calcium uptake in the "opened" membrane vesicles from arteries of NWR and SHR. For such a purpose, one should treat membrane vesicles with mild detergent, sonication or other treatments to open up the vesicles. The effects of the treatments, per se, on enzyme activity would also have to be studied to guarantee that any changes were related to opening of membrane vesicles rather than to direct effects on enzyme activities. Attempts should also be made to measure the sialic acid released by neuraminidase for the two types of fractions, before and after vesicle opening. Sialic acid is expected to be mainly on the outside of the plasma membrane. Finally, an attempt to control vesicle sidedness by enzyme activities, Ca^{++}-uptake, and availability of sialic acid residues, should be made.

(b) A comparative study should be made of the proportion of inside-out and right side-out type membrane vesicles from arteries of NWR and SHR under electron microscope by the freeze-etching technique. The protein distribution in the inner face of the cleaved membrane facing toward cytoplasm is likely to be different from that of the complementary face of the cleaved membrane. Therefore, the different patterns of protein particles on the cleaved membrane faces observed in each group of vesicles may indicate the different proportions of these two types of vesicles from either NWR or SHR arteries.

2. The second hypothesis to be considered is whether the essential difference between plasma membrane vesicles from arteries of normotensive

and hypertensive rats is that more of the latter are less permeable to Ca^{++}.

Some of the results can fit this hypothesis, e.g., decreased $^{45}Ca^{++}$-uptake in the absence of ATP, if one assumes that ATP-independent calcium uptake is due to calcium permeating through the vesicles, and then binding to the inside of the sealed vesicles, i.e., to the outside of the plasma membrane. Also the fact that the ATP-dependent calcium uptake by plasma membrane fraction from SHR arteries was greater than that from NWR can be explained as follows: The vesicles from SHR arteries are less leaky or less permeable to Ca^{++}, and therefore a greater calcium gradient develops across the membranes of the vesicles from SHR than that from NWR. The existence of such extra Ca^{++} accumulation would also explain the greater release of Ca^{++} by ionophores from SHR vesicles. These results may imply that plasma membrane of intact vessels from SHR is also less permeable to calcium than that from NWR. The difference in the ability of divalent cations to compete with $^{45}Ca^{++}$-uptake by the plasma membrane vesicles from NWR and SHR arteries can also be explained by the possibility that the vesicles from SHR arteries are less permeable to $^{45}Ca^{++}$ than those from the former.

If the proportion of right side-out and inside-out vesicles is the same in both SHR and NWR preparations, the higher $^{45}Ca^{++}$-uptake by SHR vesicles could be because they are less leaky to $^{45}Ca^{++}$ (see above). Sr^{++} and Ca^{++} at the concentration of 10 mM and 1 mM respectively may be effectively competing with that fraction of $^{45}Ca^{++}$ actively transported in both types of vesicles since the residual $^{45}Ca^{++}$ remains also the same in the two preparations. Conceivably Ba^{++} was unable to inhibit ATP-dependent binding or transport process and the effects on $^{45}Ca^{++}$-uptake were due to competition at other binding sites. The $^{45}Ca^{++}$ displaced by Ba^{++} was not much more than that bound non-specifically. If so, Ba^{++} was a better competitor for such sites in NWR vesicles and the decreased Ca^{++} permeability of SHR vesicles would have to imply improved ATP independent or non-specific binding of $^{45}Ca^{++}$. However, the greater displacement of $^{45}Ca^{++}$ by Ba^{++} in vesicles from NWR may be unrelated to Ca^{++} permeability and due to the differences in the $^{45}Ca^{++}$-binding properties of NWR and SHR vesicles.

In order to explain the effect of Mn^{++}, one would have to postulate that Mn^{++} affects mainly the ATP-independent calcium binding or affects binding only in the absence of ATP if it is complexed selectively by ATP. The results of the effects of other cations on ATP-independent Ca^{++}-uptake can be explained as follows: Sr^{++}, Ca^{++} and Mn^{++} compete with the binding sites for $^{45}Ca^{++}$ on the inner surface of vesicles, but do not affect the diffusion of $^{45}Ca^{++}$ across the membrane. They may be quite effective in displacing $^{45}Ca^{++}$-bound on the inside of the vesicles. Since membrane is less permeable to Ca^{++} in SHR vesicles, $^{45}Ca^{++}$ accumulation should be less in the inside of these vesicles. Therefore, the residual $^{45}Ca^{++}$-uptake in SHR vesicles should be less, which is to be expected. However, Ba^{++} would have to compete with $^{45}Ca^{++}$ in the diffusion process across the membrane instead of competing with $^{45}Ca^{++}$-binding inside of the vesicles since Ba^{++} inhibits $^{45}Ca^{++}$-uptake by SHR vesicles more than that of NWR vesicles. To further test this hypothesis, the following experiments are suggested:

(a) A comparative study of "rates of calcium release" after vesicles from arteries of NWR and SHR are loaded with $^{45}Ca^{++}$ in the presence of ATP, and a dilution technique is used to stop the uptake, could be informative. On diluting out both the ATP and the Ca^{++} passive leakage of $^{45}Ca^{++}$ should determine the rate of loss of tracer. The rate constant for this process should be slower in vesicles from arteries of SHR than in vesicles from arteries of NWR despite their presumed steeper Ca^{++} gradient. Further, some studies should be carried out with dilution of the Ca^{++} only, maintaining ATP concentrations in the event ATP affects passive permeability to Ca^{++}. Finally, studies should be done in which $^{45}Ca^{++}$ containing solutions are replaced by varying amounts of unlabelled Ca^{++} while ATP levels are maintained, in some cases but not in others, in order to follow the efflux of $^{45}Ca^{++}$ tracer and determine its dependence on external Ca^{++}. This would evaluate possible differences in a Ca^{++}-Ca^{++} exchange process between the two types of vesicles and their dependence on ATP.

(b) For comparative study of influx and efflux of $^{45}Ca^{++}$ by the isolated blood vessels from NWR and SHR, one should consider the possibilities of calcium binding to the inside, and outside of the muscle cells, and the problems associated with compartmental analysis (see Daniel and Janis, 1975). Shibata et al. (1975) have carried out similar studies using aorta and reported a lesser influx of $^{45}Ca^{++}$ based on the lanthanum method of Van Breemen et al. (1972). However, I have shown that $^{45}Ca^{++}$-uptake by plasma membrane vesicles is decreased in vesicles from aortae but increased in vesicles from mesenteric arteries. These studies will have to be carried out with mesenteric arteries. The validity of the techniques for estimating Ca^{++} fluxes must be carefully scrutinized.

(c) Studies of the dependence on extracellular calcium of contractions of these two types of arteries. If plasma membranes from hypertensive mesenteric arteries are less leaky to Ca^{++} than those from normotensive arteries, one would expect them to retain contractility in low Ca^{++} solution longer, to have a different dependence of their contractions (especially K^{+} contractions) on external Ca^{++} concentrations, to show a different relation between external K^{+} and contraction tension, etc.

3. The third hypothesis to be considered is whether the essential difference between plasma membrane vesicles from arteries of normotensive and hypertensive rats is that the latter are more permeable to Ca^{++}. This hypothesis has been considered because it has been strongly advocated by Somlyo and Somlyo (1970).

A decreased ATP-independent calcium uptake (1 mM) by the plasma membrane fraction from SHR arteries than that from NWR may be caused, not by lesser leakage of Ca^{++} into vesicles but instead by greater leakage out. However, this would imply that Ca^{++}-uptake in the absence of ATP was somehow dependent on a gradient of concentration across the vesicle membrane and this seems unlikely. An increased ATP-dependent calcium uptake by the plasma membrane fraction from SHR arteries as compared to that of NWR arteries may be a compensatory (indeed an over-compensatory) response to such an increase in membrane permeability to Ca^{++}. However, this would imply that the greater ATP-dependent Ca^{++}-uptake by vesicles from arteries of SHR compared to those from arteries of NWR was a result of maintenance of an increased Ca^{++} gradient or increased Ca^{++} binding inspite of a fast leak of this ion down hill. A further increase in the leak by adding ionophores to the point of abolishing the gradients in each case would eliminate any difference arising from this factor but should allow the persistence of any difference arising from ATP-dependent binding. Thus, on this hypothesis, the ionophore results would imply greater Ca^{++} transport not binding. However, there is no independent evidence that hypertensive arteries transport Ca^{++} more efficiently following a contraction compared to normotensive arteries.

The different ability of divalent cations to compete with Ca^{++}-uptake by the membrane vesicles from NWR and SHR arteries may fit this hypothesis. As discussed in the hypotheses (1) and (2), in the ATP-dependent $^{45}Ca^{++}$-uptake study, cold Ca^{++} and Sr^{++} may act on the $^{45}Ca^{++}$ active transport process, whereas Ba^{++} and Mn^{++} may act on the ATP independent $^{45}Ca^{++}$-binding process. As a result, greater inhibition of $^{45}Ca^{++}$-uptake by Ca^{++} or Sr^{++} in NWR and SHR vesicles would be expected. The greater effect of Ba^{++} on vesicles from NWR arteries is hard to explain without involving additional hypotheses. The ability of these cations to compete with $^{45}Ca^{++}$-uptake may be different in SHR and NWR vesicles. In the ATP-independent $^{45}Ca^{++}$-uptake, the differential effects of these cations on $^{45}Ca^{++}$-uptake in the two kinds of vesicles is very difficult to explain. To further test this hypothesis, the experiments suggested for the second hypothesis can be followed. In addition, since this hypothesis implies accelerated ATP-dependent membrane transport of Ca^{++} by arteries of SHR, the occurrence of this should be evaluated in intact membranes and isolated vesicles.

4. The fourth hypothesis to be considered is whether the essential

difference between plasma membrane vesicles from arteries of normotensive and hypertensive rats is that the latter retain their immature, protein synthesizing, proliferative character. If hypertension has a structural cause, i.e. increased wall thickness, then the changes associated with the proliferative activity might reflect the pathological process which underlies structural change.

So far, the physiological functions of the enzymes studied are not clear. However, the specific activities of many enzymes are increased in the plasma membrane fraction from arteries of SHR when compared to that of the same age of NWR. Also 5'-nucleotidase activity has been found to be increased in proliferative cells (Oka and Angrist, 1967). It is possible that arteries of SHR retain higher enzyme activities because they retain their immature proliferative character. This would not explain the increased activity of alkaline phosphatase in artery membranes of older SHR compared to artery membranes of younger SHR.

There is no obvious reason to predict that the proliferative character of the hypertensive arteries should be associated with an increased calcium uptake as observed in this study. However, no obvious reason excludes such an association. Similarly, there is no obvious reason why an increased proliferative activity would affect the ability of cations to compete with Ca^{++}.

In this study, I could not detect any difference in protein yield between the corresponding subcellular fractions from NWR and SHR arteries. If arteries from SHR were proliferative then a larger ER fraction might have been expected. One possibility to explain this is that the protein yield difference between NWR and SHR arteries may be small, and the method not sensitive enough to find a difference of such magnitude. Another possibility is that the hypothesis may not be true. To further test this hypothesis, the following experiments are suggested:

(a) A stereological study by electron microscopy of the relative volume occupied by the subcellular organelles in the vascular smooth muscle cell of NWR and SHR. The amounts of these organelles (e.g. plasma membrane, mitochondria and especially endoplasmic reticulum) may be related to the activity of protein synthesis.

(b) Comparative study of the rate of labelled amino acids, e.g. leucine or proline, incorporation into protein or the rate of labelled thymidine incorporation into DNA of vascular smooth muscle cells of NWR and SHR. Similar studies could be carried out in homogenates.

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